Arabidopsis *Enhanced Drought Tolerance1/HOMEODOMAIN GLABROUS11* Confers Drought Tolerance in Transgenic Rice without Yield Penalty[1][W][OA]

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Enhancing drought tolerance without yield decrease has been a great challenge in crop improvement. Here, we report the Arabidopsis (*Arabidopsis thaliana*) homodomain-leucine zipper transcription factor *Enhanced Drought Tolerance/HOMEODOMAIN GLABROUS11* (*EDT1/HDG11*) was able to confer drought tolerance and increase grain yield in transgenic rice (*Oryza sativa*) plants. The improved drought tolerance was associated with a more extensive root system, reduced stomatal density, and higher water use efficiency. The transgenic rice plants also had higher levels of abscisic acid, proline, soluble sugar, and reactive oxygen species-scavenging enzyme activities during stress treatments. The increased grain yield of the transgenic rice was contributed by improved seed setting, larger panicle, and more tillers as well as increased photosynthetic capacity. Digital gene expression analysis indicated that *AtEDT1/HDG11* had a significant influence on gene expression profile in rice, which was consistent with the observed phenotypes of transgenic rice plants. Our study shows that *AtEDT1/HDG11* can improve both stress tolerance and grain yield in rice, demonstrating the efficacy of *AtEDT1/HDG11* in crop improvement.

Drought is a major environmental stress seriously limiting plant growth and crop productivity. The ever-increasing world population and frequent global climate change challenge the world agriculture to produce enough food to feed the world (HongBo et al., 2005). To meet this challenge, it is important to improve crop yields by breeding crops with enhanced stress tolerance.

Plants have evolved many mechanisms to adapt to environmental stresses via changes at the physiological, morphological, and molecular levels (Verslues et al., 2006; Yamaguchi-Shinozaki and Shinozaki, 2006; Todaka et al., 2012). The most obvious and important mechanisms for plants to cope with dehydration stress include maximization of water uptake by deep and extensive root systems and/or minimization of water loss by stomatal closure and reduction of stomatal density, as shown by the model plant Arabidopsis (*Arabidopsis thaliana*; Yu et al., 2008).

Root system architecture and root distribution are key determinants of the ability of a plant to uptake nutrients and water to support shoot growth in drought conditions (Robinson, 1994; Lynch, 1995). It is generally believed that roots can sense changes in abiotic factors such as soil water status (Comstock, 2002), soil texture, and nutrient composition (Masle and Farquhar, 1988; López-Bucio et al., 2003; Schachtman and Shin, 2007). Root-sourced signals are transported via the xylem to leaves as soil become dry and result in reduced water loss and decreased leaf growth (Schachtman and Goodger, 2008). Root-specific expression of a cytokinin-degrading cytokinin oxidase/dehydrogenase (*CKX*) genes in Arabidopsis and tobacco (*Nicotiana tabacum*) allows the production of transgenic plants with an enlarged root system and enhanced drought tolerance (Werner et al., 2010). Rice (*Oryza sativa*) varieties with well-developed root systems have an advantage to grain yield under stress conditions (Price et al., 1997; Serraj et al., 2009).

The majority of plant transpiration occurs through stomatal pores (Taiz and Zeiger, 2010). Thus, transpirational water loss through the stomata is a key determinant of drought tolerance. Plant transpiration rate can be regulated by both stomatal movement (opening and closing) and stomatal density (Hetherington and Woodward, 2003; Lake and Woodward, 2008). Stomatal aperture is influenced by many factors, such as light, phytohormones (e.g. abscisic acid [ABA]), humidity, calcium ion, potassium ion, nitric oxide, and hydrogen peroxide (Desikan et al., 2004;

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Shimazaki et al., 2007; Kim et al., 2010). A strong negative correlation between stomatal size and stomatal density was observed, and plants with low stomatal density may be well suited to growth under water-scarce environments than plants with higher stomatal density (Doheny-Adams et al., 2012). Two receptor-like kinases, *ERECTA* and *O. sativa* Stress-Induced Protein Kinase1, were reported to affect stomatal density and drought tolerance in Arabidopsis and rice, respectively (Masle et al., 2005; Ouyang et al., 2010).

Stress-induced genes function not only in protecting cells from stress, but also in regulating genes for signal sensing, perception, and transduction in the stress response. Products of these genes can be classified into two groups (Kreps et al., 2002; Seki et al., 2002). The first group includes genes encoding metabolites or osmoprotectants that probably function to avoid cellular injury, such as key enzymes for osmolyte biosynthesis, Late Embryogenesis Abundant (LEA) proteins, and detoxification enzymes. However, overexpressing these genes not only improves drought tolerance, but also impairs plant growth, even in the absence of stress (Holmstrom et al., 1996; Abebe et al., 2003). The second group contained various transcription factors involved in further regulation of signal transduction and transcription control. These transcription factors, such as APETALA2/Ethylene Responsive Element Binding Protein family, C-repeat Binding Factor/Dehydration Responsive Element Binding factor family, v-mybavian myeloblastosis viral oncogene homolog/myelocytomatosis family, NAM, ATAF, and CUC transcription factor family, zinc finger, plant nuclear factor Y (NF-Y) B subunits family, and Basic Leucine Zipper family, play important roles in plant stress responses (Umezawa et al., 2006; Nelson et al., 2007; Takasaki et al., 2010; Yang et al., 2010). Studies of these transcription factors show promise for commercially improving drought tolerance of crops through genetic engineering. Nonetheless, ectopic overexpression of these genes is frequently associated with retarded growth and yield penalty and thus may limit its commercial applications.

In this work, we overexpressed *Enhanced Drought Tolerance1/HOMEODOMAIN GLABROUS11* (*AtEDT1/HDG11*) in rice and demonstrated that not only stress tolerance to drought, but also yield of the transgenic rice under both normal and drought conditions were significantly improved, showing the commercial potential of this gene to enhance drought tolerance and improve yield of rice. Our results also demonstrate that the *AtEDT1/HDG11*-mediated drought tolerance mechanism and biomass enhancement are conserved in monocot rice, implicating a broad application spectrum of recipient crops.

RESULTS

AtEDT1/HDG11 Significantly Improves Drought Tolerance of Transgenic Rice

Drought tolerance during seedling growth period is important for rice plant establishment in areas where dry weather overlaps with rice seedling growth. We generated transgenic rice overexpressing *AtEDT1/HDG11* (Supplemental Fig. S1) and carefully tested the homozygous transgenic lines for drought tolerance at the seedling stage. Before drought stress treatment, no obvious difference was observed between the Zhonghua11 (ZH11) control and the transgenic lines (Fig. 1A). After 5 d of water withholding, the transgenic plants showed much delayed leaf rolling compared with the wild-type ZH11 (Fig. 1, A and B). After 9 d of drought treatment and subsequent recovery for 8 d (Fig. 1, A–D), majority of the control line never recovered and only 16.7% survived. By contrast, the transgenic lines exhibited a significantly higher survival ratio, ranging from 70.4% to 100% (Fig. 1B). These results demonstrate that *AtEDT1/HDG11* can significantly improve drought tolerance of rice seedlings.

Although drought stress can affect the growth and development of rice at any time during its life cycle, flowering and grain-filling periods are most sensitive to drought. Drought stress during reproductive phase directly results in a loss of grain yield (Oh et al., 2009). To evaluate the drought tolerance at reproductive stage, the *AtEDT1/HDG11* transgenic lines and ZH11 control were subjected to drought stress at the early flowering stage. During the process of drought stress, the *AtEDT1/HDG11*-overexpressing lines showed 1 d of delayed leaf rolling morphology compared with the ZH11 control (Fig. 1C). Consistent with this result, the water loss of detached flag leaves of transgenic rice was significantly slower than that of the ZH11 plants (Fig. 1D).

AtEDT1/HDG11 Significantly Increases Grain Yield with Improved Yield Components in Transgenic Rice

Because grain yield is the ultimate parameter for evaluation of drought tolerance of crops, we carefully evaluated the *AtEDT1/HDG11* transgenic line 2-56 in the greenhouse for improved yield components. Tillering in rice is one of the most important agronomic traits related to grain production (Li et al., 2003). We therefore checked the tiller number of 6-week-old plants. Figure 2A shows that transgenic lines 2-56 and 14-16 had more tillers than ZH11. On average, the transgenic plant had one more tiller than the control at this stage. At mature stage, tiller number of transgenic line 2-56 grown in the field also increased 12.6% over the ZH11 control (Fig. 2B). Consistent with these results, greenhouse-grown 2-56 also had significantly increased tiller number (Table I). Increased tiller number is apparently one contributor to the increased yield.

The transgenic line 2-56 had larger panicle than ZH11 control (Tables I and II; Fig. 2, C and D). The increased panicle size of the transgenic rice (Fig. 2C) was largely contributed by the increased number of primary branches and secondary branches (Fig. 2D).

The filled grain number per panicle of the line 2-56 increased 12.95%, and the grain yield per plant increased 5.51% under well-irrigated conditions compared with that

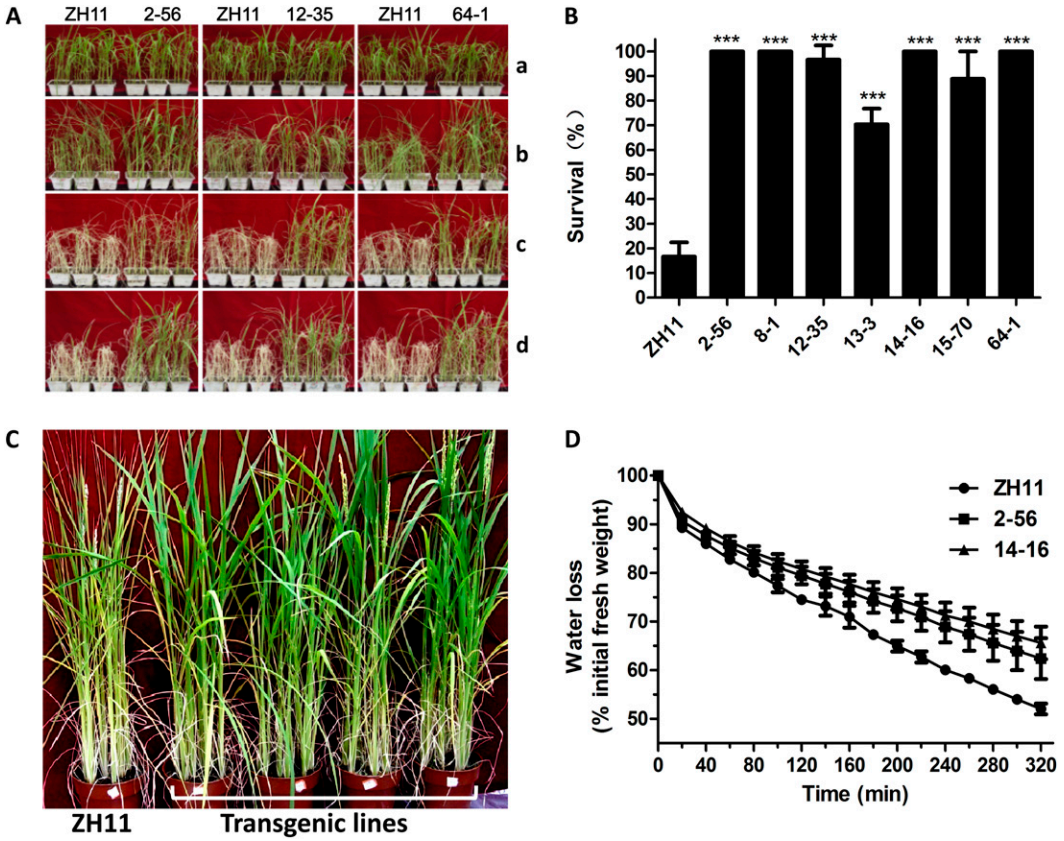

Figure 1. *AtEDT1/HDG11* improves drought tolerance of transgenic rice. A, Drought treatment of 3-week-old T3 transgenic seedlings in greenhouse. a, Before drought treatment. b, Drought for 5 d. c, Drought for 9 d and then recovery for 3 d. d, Drought for 9 d and then recovery for 8 d. B, Survival after 9 d of drought treatment and 8 d of recovery. Values are mean ± SD ($n = 27$ plants, $^{***}P < 0.001$). C, The appearance of transgenic plants and ZH11 control during drought stress at flowering stage in the greenhouse. Plants in same size containers were treated without water for 5 d. D, Water loss rate of detached flag leaves. Each data point represents the mean of duplicate measurements.

of the control (Tables I and II). A much more significant difference was observed after a 5-d drought stress during the flowering stage. The filled grain number per panicle of 2-56 was 77.39% higher than that of ZH11. Correspondingly, the seed-setting rate and grain yield per plant increased 68.62% ($P < 0.001$) and 65.17% ($P < 0.01$), respectively (Tables I and II). To gain further insights into the mechanism of the increased seed setting of 2-56, pollen viability under normal and drought stress conditions was examined. No difference between ZH11 and 2-56 was observed under normal conditions; however, after 4 d of drought treatment, pollen viability of ZH11 decreased by about 10% compared with 2-56 (Fig. 2, E and F). The higher pollen viability of transgenic rice might partially contribute to the increased seed setting and grain yield.

Increased Grain Yield of *AtEDT1/HDG11*-Expressing Transgenic Rice under Both Normal and Drought Stress Conditions in Field Trials

Transgenic lines 2-56 and 14-16 (homozygous T5) were chosen for field trials as described in "Materials and Methods." For the evaluation under drought stress, the field trials were carried out from November to April in Hainan Island, China, where rainfall is scarce during the entire growing season. For evaluation under normal conditions, the field trials were carried out in Hefei, Anhui Province, China. From 2007 to 2010, we performed multilocation field trials by two independent research groups. The results in Figure 3A show that transgenic lines produced significantly higher yields than ZH11 control either under normal conditions or under drought conditions. The relative yield increase was greater than 16% under all conditions tested. Figure 3B shows the ZH11 control and transgenic line 2-56 grown side by side in a drought stress trial on Hainan Island. Figure 3C shows the typical ZH11 and transgenic 2-56 plants from the drought stress trial with apparent differences in plant height and biomass. The increased grain yield was largely contributed by larger panicle size and higher seed-setting rate (Table II), consistent with the greenhouse results. These results demonstrate that *AtEDT1/HDG11* cannot only enhance drought tolerance in transgenic rice, but also increase grain yield, making

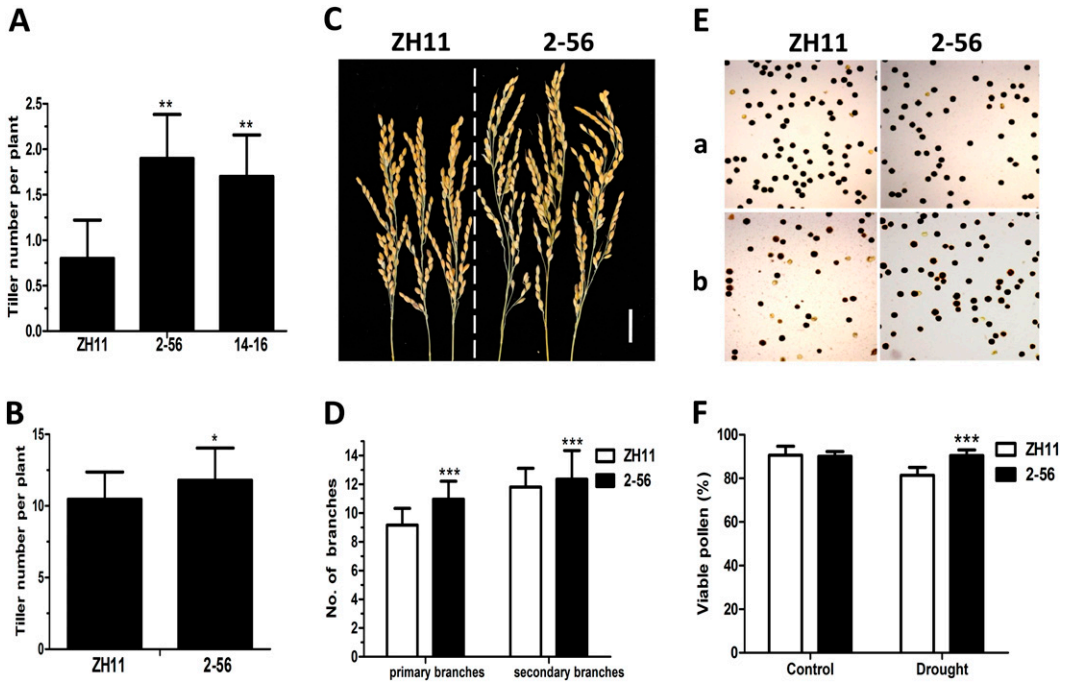

Figure 2. Improved yield components in transgenic rice. A and B, Comparison of tiller number. Tiller number of 6-week-old plants (A) or plants at mature stage (B). Twenty plants were used for each line. Values are mean ± SD (**$P < 0.01$). C, Comparison of panicles of the transgenic rice and ZH11. Bar = 5.5 cm. D, Number of primary and secondary branches per panicle. Values are mean ± SD ($n = 30$, ***$P < 0.001$). E, Pollen viability between transgenic and control plant. Mature pollens under normal conditions (a) and after 4 d of drought stress (b) stained with iodine-potassium iodide. F, Viable pollen ratio between transgenic and control plants before and after drought treatment. Values are mean ± SD (***$P < 0.001$).

a huge stride toward commercial utilization of this technology.

Reduced Leaf Stomatal Density, Enlarged Stomatal Size, and Flag Leaf Area in the *AtEDT1/HDG11* Transgenic Rice

Adaxial stomatal density and size of topmost fully expanded leaf at the young seedling growth stage and flag leaf at the flowering stage were determined by leaf surface imprint method. At seedling stage, the average stomatal density of the transgenic lines 2-56 and 14-16 was reduced by 7.4% and 21.6% compared with that of the ZH11 control, respectively (Fig. 4A). While the stomatal density was reduced, the stomatal size was increased in the *AtEDT1/HDG11*-overexpressing lines at seedling stage (Fig. 4B). The average stomatal length and width of transgenic lines increased by 3.8% to 12.4% and 12.1% to 22.4%, respectively. Similar phenotype was also detected in flag leaf of 2-56 at flowering stage (Supplemental Fig. S2). Although stomatal density was changed, stomatal index was not altered. These results were consistent

Table I. *Grain yield and yield components under normal and drought stress conditions in greenhouse*

Values are mean ± SD ($n > 25$). Percentage symbol in the last column indicates percentage increase or decrease relative to ZH11 control.

Lines	No. of Tillers per Plant	Panicle Number per Plant	Panicle Length	No. of Grains per Panicle	Filled Grains per Panicle	Seed-Setting Rate	1,000-Grain Weight	Grain Yield per Plant
			cm			*%*	*g*	*g*
Normal (control)								
ZH11	10.67 ± 2.16	10.52 ± 2.08	19.66 ± 1.16	102.23 ± 6.66	98.00 ± 5.94	96.19 ± 1.14	24.75 ± 0.04	27.77 ± 0.40
2-56	11.32 ± 2.36[a]	11.05 ± 1.88[a]	21.19 ± 2.26[a]	117.00 ± 14.37[b]	110.69 ± 14.64[b]	94.58 ± 3.80	24.88 ± 0.07	29.30 ± 0.81[a]
%	6.1	5.05	7.78	14.45	12.95	–1.67	0.54	5.51
Drought								
ZH11	9.07 ± 1.67	8.79 ± 2.54	20.03 ± 1.65	105.04 ± 14.13	24.88 ± 8.84	23.42 ± 7.10	20.55 ± 0.24	4.63 ± 0.57
2-56	11.08 ± 1.75[a]	9.58 ± 1.50[a]	21.20 ± 1.65[b]	114.46 ± 11.44[a]	44.14 ± 11.44[c]	38.62 ± 9.31[c]	20.43 ± 0.11	7.65 ± 0.75[b]
%	11.15	9.08	5.84	8.97	77.39	68.62	–0.57	65.17

[a]$P < 0.05$. [b]$P < 0.01$. [c]$P < 0.001$.

Table II. *Improved yield components of the transgenic line 2-56 in 2007–2008 field trial*

Treatment		Total No. of Seeds per Panicle	No. of Filled Seeds per Panicle	Seed Setting	Yield per Panicle
				%	g
Normal condition	ZH11	106.21	71.77	67.69	1.67
	2-56	132.09[a]	106.81[b]	80.92[a]	2.33[a]
Drought condition	ZH11	60.10	25.07	41.72	0.49
	2-56	80.92[a]	49.82[b]	61.53[b]	1.11[b]

[a]$P < 5\%$. [b]$P < 1\%$.

with our previous study in Arabidopsis and transgenic tobacco (Wang et al., 2007; Yu et al., 2008). The reduced stomatal density apparently contributes to the reduced rate of water loss of transgenic plants.

The top three leaves, especially flag leaf, of rice contribute most to grain production (Ray et al., 1983). Length and width of flag leaf of ZH11 and two transgenic lines (2-56 and 14-16) at flowering stage in the field were measured. The flag leaf area was calculated according to Yoshida et al. (1976). The results in Figure 4, C and D, show that the transgenic lines have significantly larger flag leaf size and area than ZH11, which should enhance the photosynthetic capacity.

Photosynthetic Rate and WUE Are Improved in *AtEDT1/HDG11* Transgenic Plants

It is well known that stomatal density can affect CO_2 and water exchange (Hetherington and Woodward, 2003). We thus measured photosynthesis and Water Use Efficiency (WUE) of ZH11 control and *AtEDT1/HDG11*-overexpressing plants at reproductive stage in the field. Interestingly, photosynthetic rate of the flag

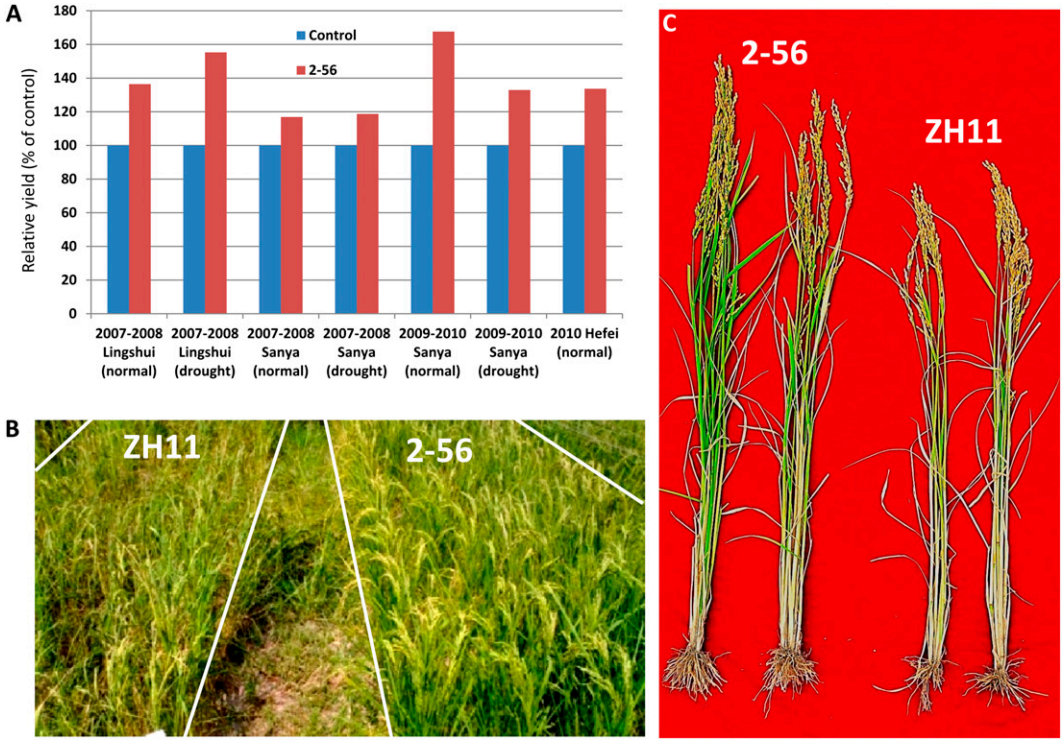

Figure 3. *AtEDT1/HDG11* improves grain yield in field trials. A, Yield of multiyear and multilocation field trials. Homozygous T5 lines were used for multilocation (Sanya or Lingshui, Hainan Province, China; Hefei, Anhui Province, China) field trials by two independent research groups as described in "Materials and Methods" from 2007 to 2010. Yield of 2-56 transgenic line was normalized to that of the ZH11 control in each trial and presented as the percentage of the control. B, Transgenic line 2-56 and ZH11 control in a drought stress field trial in Lingshui, Hainan Island, China. C, Typical ZH11 and transgenic 2-56 plants from the drought stress field trial in Lingshui.

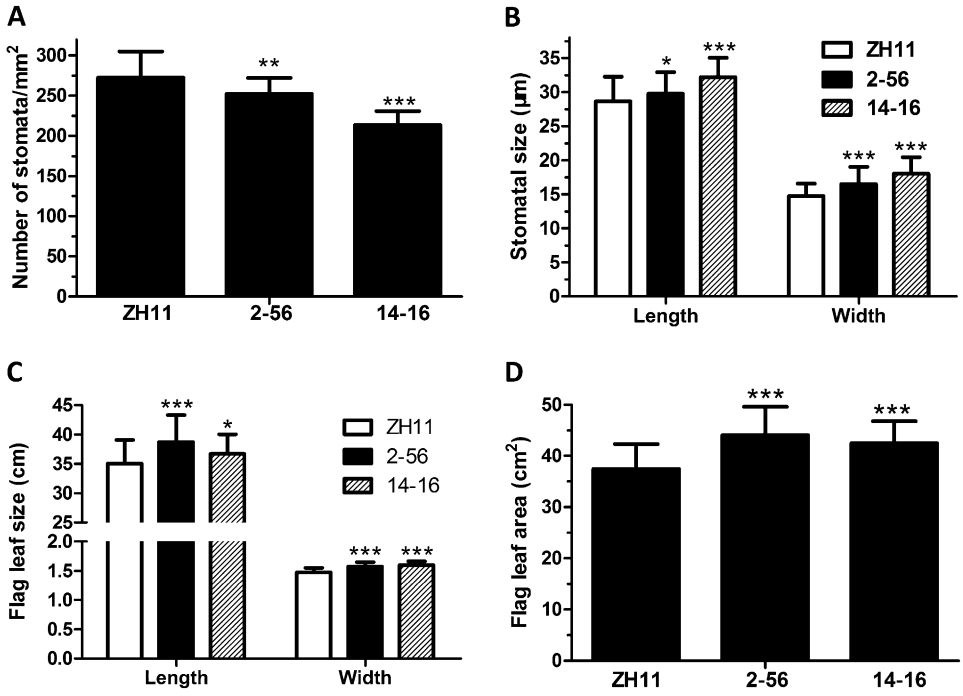

Figure 4. Reduced leaf stomatal density, enlarged stomatal size, and flag leaf area in the *AtEDT1/HDG11* transgenic rice. A and B, Comparisons of stomatal density (A) and stomatal dimension (B) in the ZH11 control and the transgenic plants at seedling stage. Three leaves were sampled for each plant, and 10 plants were sampled for both the control and the transgenic lines. Values are mean ± SD ($n = 300$, $^*P < 0.05$, $^{**}P < 0.01$). C, Width and length of the flag leaf. Fifty flag leaves from 30 plants of the transgenic lines and ZH11 were used for measurement, respectively. Values are mean ± SD ($^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$). D, The flag leaf area. Leaf area was calculated according to Yoshida et al. (1976): leaf area = leaf length × leaf width × 0.725. Values are mean ± SD ($^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$).

leaf was increased 11.1% to 17.5% in the transgenic plants (Fig. 5A). While transpiration rate was reduced in transgenic lines, but only statistically significant in 2-56 (Fig. 5B), stomatal conductance was reduced in both transgenic lines 2-56 and 14-16 (Fig. 5C). Consequently, WUE of all the transgenic lines is significantly higher than ZH11 control (Fig. 5D). These results indicate that *AtEDT1/HDG11* can enhance photosynthetic efficiency and increase WUE in rice, consistent with our previous observation in Arabidopsis mutant *edt1* (Yu et al., 2008).

Larger Root System in *AtEDT1/HDG11* Transgenic Rice

A deep and thick root system is able to extract water in deep soil and is considered important in determining drought tolerance in upland rice (Kavar et al., 2008). Therefore, we examined the root architecture of *AtEDT1/HDG11* transgenic rice at seedling stage. The transgenic plants at seedling stage showed a larger root system than ZH11 control with markedly increased root length and number (Fig. 6, A–C) as that in *edt1* mutant (Yu et al., 2008). Consequently, dry root biomass of the two tested transgenic lines 2-56 and 14-16 was 46.1% and 71.3% higher, respectively, than that of the control plants (Fig. 6D). Transgenic plants grown in the field also developed a larger root system compared with ZH11 (Fig. 6E). The altered root architecture of transgenic rice would enhance the uptake of water, positively contributing to drought tolerance.

ABA, Pro, Soluble Sugar, and Superoxide Dismutase Activity Are Increased in Transgenic Rice

ABA plays important roles in plant drought response. To determine whether ABA level was changed in the transgenic plants, ABA content in leaves of both the transgenic and ZH11 control plants were measured. Under normal conditions, the ABA content was higher in the transgenic lines than that in ZH11, especially in 2-56. After 5 d of drought treatment, ABA content of both the transgenic and ZH11 was increased. However, the transgenic plants 2-56 and 14-16 had 37.7% and 24.4% higher levels than that of ZH11, respectively (Fig. 7A).

Meanwhile, Pro and soluble sugar, two common compatible osmolytes in higher plants, were measured before and after 7 d of drought stress. The Pro content of the transgenic plants was higher than that of the control under normal conditions, but no significant difference was observed in soluble sugar before drought stress. However, after 7 d of drought treatment, a significant increase of both Pro and soluble sugar content was observed in the transgenic plants compared with that in the control (Fig. 7, B and C).

Superoxide dismutases (SODs) are important antioxidant enzymes responsible for scavenging superoxide radicals in plants (Kliebenstein et al., 1998). SOD activity assays showed a significantly higher activity in the transgenic plants than in the control after drought stress (Fig. 7D), indicating an enhanced capability to scavenge reactive oxygen species in the transgenic plants.

Expression Profiling Analysis of Flag Leaf at Flowering Stage

To explore the molecular mechanisms of drought tolerance underlying the *AtEDT1/HDG11*-overexpressing rice, Illumina digital gene expression (DGE) tag profiling was performed to determine the differential gene expression between ZH11 and the 2-56 transgenic line in

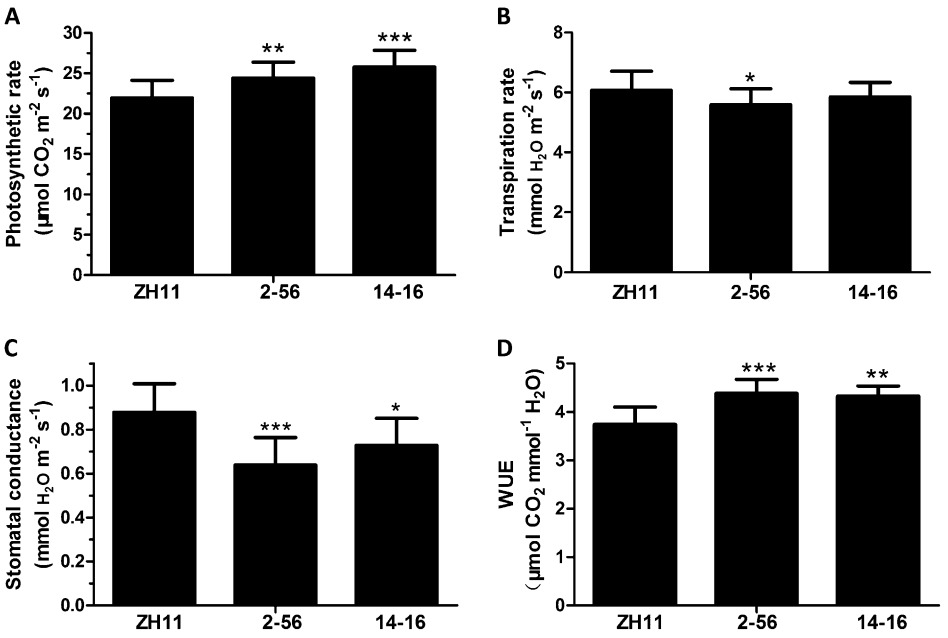

Figure 5. Photosynthetic rate and WUE are improved in *AtEDT1/HDG11* transgenic plants in the field. A to D, Comparisons of photosynthetic rate (A), transpiration rate (B), stomatal conductance (C), and WUE (D) between the ZH11 control and the transgenic plants. Three measurements were carried out for each plant, and 10 plants were used for each line. Values are mean ± SD (*$P < 0.05$, **$P < 0.01$).

flag leaf at flowering stage under both normal and drought conditions. Under normal conditions, 730 genes were up- or down-regulated by at least 2-fold in 2-56 (2-56N) compared with ZH11 (ZH11N). After drought treatment, 1,412 and 1,088 genes were detected with greater than 2-fold difference of transcript level in ZH11 (ZH11D) and 2-56 (2-56D) compared with ZH11N, respectively (Table III). We also found 1,003 differentially expressed genes (DEGs) between 2-56 (2-56D) and ZH11 (ZH11D) after drought treatment.

Cluster analysis revealed that expression pattern for all genes significantly expressed ($P < 0.01$, uncorrected)

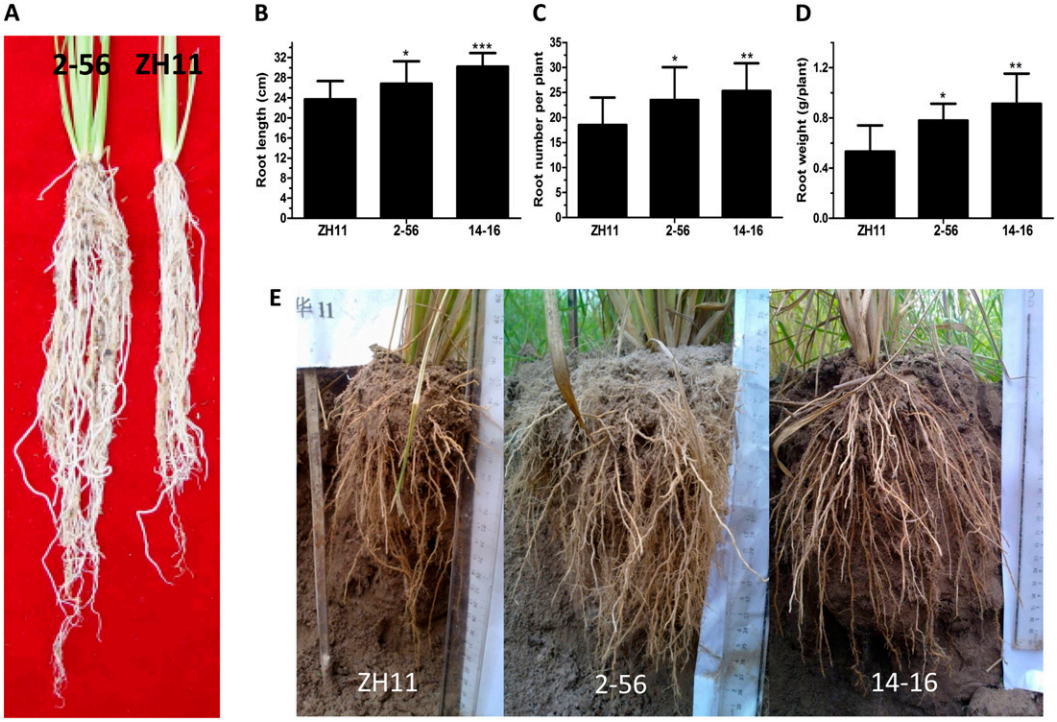

Figure 6. Larger root system of *AtEDT1/HDG11* transgenic rice. A, Root system of 6-week-old control and transgenic plants. B to D, Comparison of the longest root length (B), root number per plant (C), and root weight per plant (D). Twenty 6-week-old plants were used for each line. Values are mean ± SD (*$P < 0.5$, **$P < 0.01$, ***$P < 0.001$). E, Comparison of the root system at mature stage under drought conditions in the field.

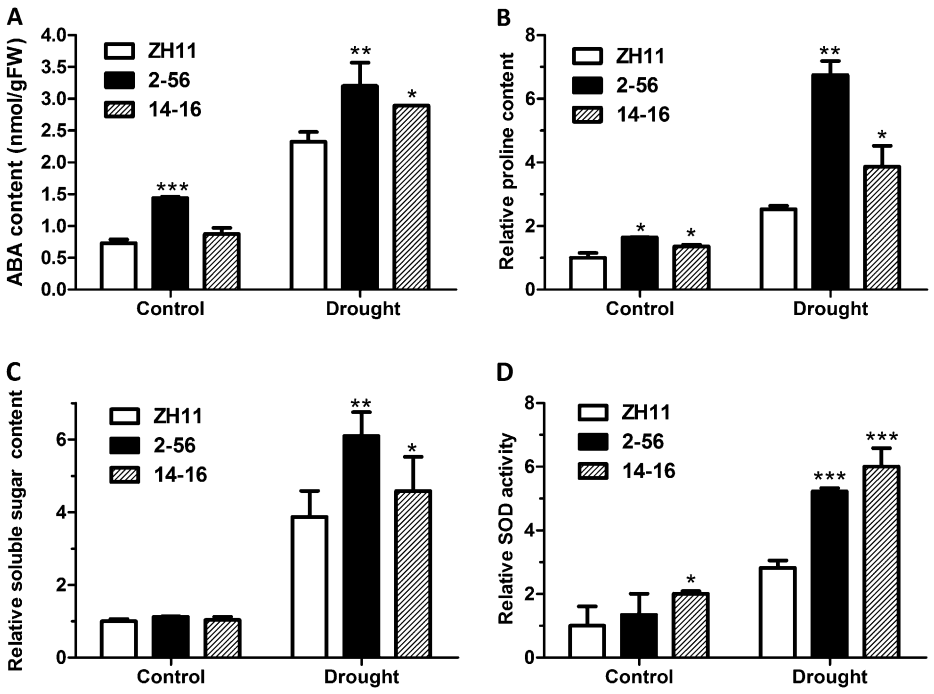

Figure 7. ABA, Pro, soluble sugar, and SOD activity are increased in transgenic rice. A to D, ABA content (A), Pro content (B), soluble sugar (C), and SOD activities (D) in the leaves of 2-week-old transgenic and ZH11 control plants with or without drought treatments. Values are mean ± SD of three independent experiments (*$P < 0.05$, **$P < 0.01$). FW, Fresh weight.

in 2-56D compared with ZH11N did not significantly overlap with ZH11D compared with ZH11N, as well as 2-56N compared with ZH11N (Fig. 8A). These results demonstrated that *AtEDT1/HDG11* had a significant impact on global gene expression profile in rice, implicating that novel mechanisms may underlie the drought tolerance of the transgenic rice. We also compared the DEGs of 2-56N, 2-56D, and ZH11D compared with ZH11N. Venn diagram results indicate that only a number of DEGs overlapped between 2-56N/ZH11N, 2-56D/ZH11N, and ZH11D/ZH11N. Among the 657 up-regulated genes and 431 down-regulated DEGs of 2-56D/ZH11N, 210 (32%) and 210 (48.7%) genes exclusively appeared in 2-56D/ZH11N but not in ZH11D/ZH11N, respectively (Fig. 8, B and C).

In addition to these DEGs, many well-known stress-related genes were found up-regulated in both ZH11D and 2-56D (Table IV). Most of these genes showed higher transcript level in 2-56 compared with ZH11 under normal conditions as well as under drought conditions. To confirm the results of DGE, nine stress-related genes were selected for quantitative real-time Reverse Transcription (RT)-PCR. Most of these genes (seven out of nine) showed higher expression level in 2-56N compared with ZH11N. However, seven genes, excluding LEA3-1 and *OsNAC5*, showed significantly higher expression level in 2-56D than in ZH11D ($P < 0.05$; Supplemental Fig. S3). These results suggested that overexpression of *AtEDT1/HDG11* elevated the transcript levels of many stress-resistance genes under drought stress conditions, thus improving the drought tolerance of the transgenic rice. Among the genes with significantly higher induction in 2-56D, quite a few encode stress-responsive transcription factors, such as *SNAC1*, *OsbZIP23*, and *SNAC2*, indicating that *AtEDT1/HDG11* may indirectly regulate the expression of a large number of stress-responsive genes. Moreover, *O. sativa* NINE-CIS-EPOXYCAROTENOID DIOXYGENASE3 (*OsNCED3*), which is the key gene of ABA biosynthesis, was found significantly up-regulated in both 2-56N and 2-56D. This result is consistent with higher level of ABA content in 2-56 compared with ZH11 (Fig. 7A).

We further classified DEGs based on Gene Ontology through the Web tool DAVIA (Huang et al., 2008). All genes in the rice genome were used as background for significance testing. We found that DEGs between 2-56N and ZH11N were significantly enriched in biological process categories involved in photosynthesis (1.11E–08), photorespiration (5.95E–05), oxidation reduction (1.78E–04), and carbon fixation (3.58E–04; Supplemental Table S1). The cluster of photosynthesis-related genes had the highest enrichment score (Supplemental Table S2). After

Table III. *Number of DEGs*

2-56N/ZH11N refers to DEGs between 2-56 and ZH11 under normal conditions. ZH11D/ZH11N refers to DEGs between ZH11 under drought conditions and ZH11 under normal conditions. 2-56D/ZH11N refers to DEGs between 2-56 under drought conditions and ZH11 under normal conditions. 2-56D/ZH11D refers to DEGs between 2-56 and ZH11 under drought conditions.

Type	No. of DEGs (≧2)
2-56N/ZH11N	730
ZH11D/ZH11N	1,412
2-56D/ZH11N	1,088
2-56D/ZH11D	1,003

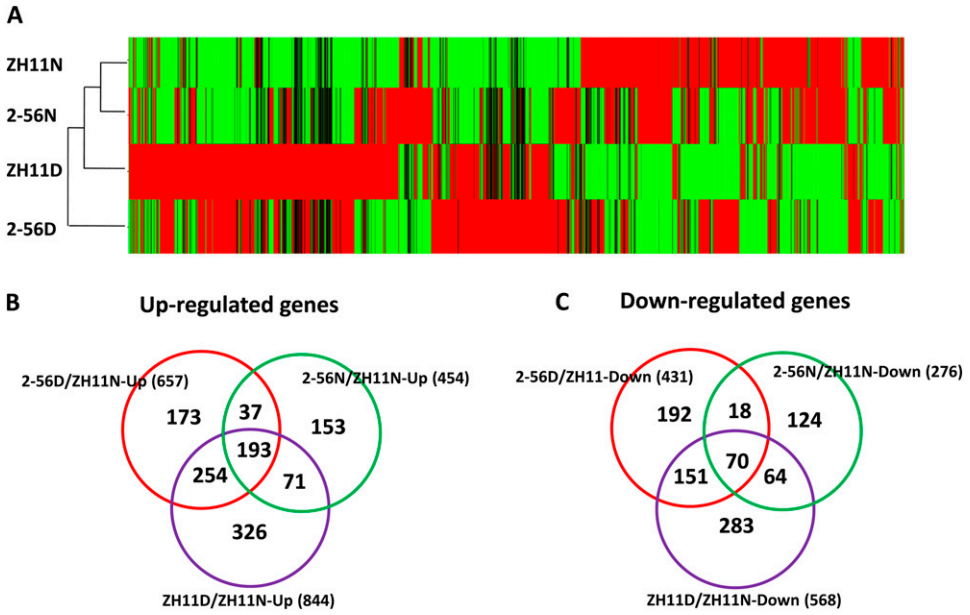

Figure 8. Overview of serial analysis of DEGs of 2-56D, ZH11D, and 2-56N compared with ZH11N. A, Hierarchical clustering analysis of all DEGs based on expression data. B and C, Venn diagram of up- and down-regulated DEGs of 2-56D/ZH11N, ZH11D/ZH11N, and 2-56N/ZH11N. D refers to drought treated, and N refers to normal control.

drought treatment, similar results were also obtained. The most enriched gene categories in DEGs between 2-56D and ZH11D were also related to photorespiration (8.16E-04), carbon fixation (4.59E–03), regulation of transcription (5.72E–03), and oxidation reduction (5.62E–02; Supplemental Table S3). These results implied that *AtEDT1/HDG11* had a significant influence on the expression of genes related to photosynthesis and oxidation reduction, consistent with the results of increased photosynthesis and grain yield.

DISCUSSION

Improved Drought Resistance of *AtEDT1/HDG11*-Overexpressing Transgenic Rice Is Attributed to Multiple Determinants

In this study, we evaluated *AtEDT1/HDG11* in transgenic rice. Our results provided solid evidence that overexpressing *AtEDT1/HDG11* was able to not only improve drought tolerance, but also increase grain yield of rice, demonstrating that it is a promising candidate gene for crop improvement. The drought tolerance phenotype of *AtEDT1/HDG11* transgenic rice plants is contributed by a collection of beneficial factors observed in the overexpressors.

First, the overexpression of *AtEDT1/HDG11* in rice conferred a more extensive root system (Fig. 6). The overall size and maximum depth of the rice root system are positively related to field drought tolerance (Ekanayake et al., 1985; Price and Tomos, 1997). Second, expression of *AtEDT1/HDG11* in rice resulted in decreased transpiration rate and enhanced photosynthesis (Fig. 5), thus improving its WUE. Reduced stomatal density and conductance could be attributed, at least in part, to changes in the transpiration rate. Third, the higher Pro and soluble sugar content, as well as increased SOD activity detected in the transgenic plants after drought stress (Fig. 7), indicated that they were better protected from oxidative and osmotic damages. Fourth, the ABA content in leaf is increased in transgenic rice compared with that of ZH11 (Fig. 7). Increased ABA not only triggers the closing of stomata to control transpiration (Schroeder et al., 2001), but more importantly, may expand the capacity of stress response.

At last, the elevated transcript levels for several stress tolerance genes in *AtEDT1/HDG11* transgenic rice under both normal and drought stress conditions (Table IV; Supplemental Fig. S3), including a few transcription factors reported to confer drought tolerance in plants, should contribute to the improved drought tolerance. Taken together, the overexpression of *AtEDT1/HDG11* triggered multiple determinants that improve the ability of both water conservation and water accessibility in the transgenic rice, as well as cellular tolerance to stresses.

Grain Yield Increase of *AtEDT1/HDG11* Transgenic Rice with Enhanced Drought Tolerance Is Contributed by Increased Photosynthesis and Multiple Improved Yield Components

Crop production is limited by a combination of abiotic and biotic stresses. Drought is the most important abiotic stress that severely restricts crop production (Boyer, 1982; Rockstrom and Falkenmark, 2000). Recent studies suggested that overexpression of stress-related genes may improve drought tolerance in rice to some extent in laboratory or greenhouse conditions (Dubouzet et al., 2003; Park et al., 2005; Chen et al., 2008; Hou et al., 2009; Huang et al., 2009b; Cui et al., 2011; Gao et al., 2011; Yang et al., 2012; Zou et al., 2012), with very few reports to date on field testing (Xiao et al., 2007; Xiang et al., 2008). However, a gap still exists between the results in the laboratory and the application of these techniques to the staple

Table IV. *Expression of stress-related genes identified by DGE tag profiling*

RPKM refers to per kilobase of exon model per million mapped reads, which reflects the expression level of the gene. D, S, C, A, and H refer to response to drought, salt, cold, ABA, and high temperature stresses, respectively. GA, gibberellins; ET, ethylene; JA, jasmonic acid.

ID	Description	RPKM				Stress Response	References
		2-56D	2-56N	ZH11D	ZH11N		
Os03t0815100-01	*NAC1* transcription factor	119	32	77	19	D, S, C, A	Hu et al., 2006
Os02t0766700-01	*OsbZIP23*	63	24	54	12	D, S	Xiang et al., 2008
Os05t0542500-02	*OsLEA3-1*	22	0	27	1	D, S, A	Xiao et al., 2007
Os01t0869900-01	SNF1-type serine-threonine protein kinase	35	15	15	11	D, S	Diédhiou et al., 2008
Os02t0527300-01	Heat shock transcription factor 3	80	21	24	13	H	Wang et al., 2009
Os03t0655400-01	Similar to water stress-induced protein dehydrin	846	156	499	262	D	
Os02t0181300-01	*OsWRKY71* transcription factor	212	68	149	65	GA	Xie et al., 2006
Os06t0216300-01	*OsOPR1* (12-oxo-phytodienoic acid reductase gene)	43	10	5	2	D, S, A	Agrawal et al., 2003
Os03t0645900-00	*OsNCED3*	21	1	5	0	D	Hwang et al., 2010
Os09t0287000-01	*Sub1C* (*Submergence1C*), APETALA2/ERF domain ethylene-responsive transcriptional factor	50	15	33	21	ET, GA	Peña-Castro et al., 2011
Os01t0135700-01	*OsCML16* (calmodulin-like protein16)	32	12	28	9		Boonburapong and Buaboocha, 2007
Os05t0455500-02	Δ-1-pyrroline-5-carboxylate synthetase (*OsP5CS*)	62	24	55	23	D, C, H, S, A	Hien et al., 2003
Os07t0515100-02	Calcium-dependent protein kinase, *OsCDPK2*	115	63	58	56		Frattini et al., 1999
Os01t0884300-01	*SNAC2*	343	135	267	126	D, C, S, A	Hu et al., 2008
Os11t0126900-01	*SNAC10*	36	6	45	4	D, S, A	Jeong et al., 2010
Os11t0184900-02	*OsNAC5*	22	7	26	5	D, S, C, A, JA	Takasaki et al., 2010
Os12t0586100-01	SNF1-type Ser-Thr protein kinase, *SAPK8*	155	77	29	143		
Os11t0454300-01	Responsive to ABA21 (*RAB21*)	19	0	30	0	D, A	Mundy and Chua, 1988
Os02t0669100-01	Dehydration-stress inducible protein1	110	26	122	25	D	
Os09t0486500-04	*OsSAP1*	46	15	85	19	D, S	Giri et al., 2011
Os08t0504700-01	*OsSAP11*	80	59	144	69	D, S	Giri et al., 2011

crops in the field. One of the problems is that the constitutive overexpression of stress-related genes often causes abnormal development and thus a loss in productivity (Kasuga et al., 1999; Priyanka et al., 2010; Dubouzet et al., 2003; Nakashima et al., 2007; Hsieh et al., 2002). The improvement of drought tolerance should be achieved without a parallel limitation of plant growth and yield potential (Cattivelli et al., 2008). There is an urgent need for developing plants that are tolerant to multiple stresses yet maintain high yields under normal conditions.

In this study, we demonstrated that overexpression of *AtEDT1/HDG11* in rice not only improved its drought tolerance, but also increased the grain yield under both normal and drought stress conditions (Figs. 1 and 3; Tables I and II). Based on our results, *AtEDT1/HDG11*-improved grain yield of rice is contributed at least by the following factors. First, the transgenic lines are more tolerant to drought because they are better protected from oxidative and osmotic damages and more responsive to stress due to elevated levels of ABA, Pro, soluble sugars, and SOD activity (Fig. 7). Thus, the transgenic lines perform better on growth and yield under drought stress compared with ZH11 control.

Second, the transgenic rice has a larger panicle size and more tillers than ZH11 (Fig. 2, A–D; Tables I and II). Panicle size and tiller number directly contribute to grain yield. *AtEDT1/HDG11* is likely involved in development during the reproductive phase in the wild-type Arabidopsis because its expression pattern is confined to flowers, flower buds, and immature siliques (Yu et al., 2008). Moreover, its amino acid sequence shows extensive homology to the known development regulators of HD-START proteins (Nakamura et al., 2006). It is possible that *AtEDT1/HDG11* is involved in the development of panicle in rice. The underlying mechanism is currently unknown but interesting to explore in the future.

Third, the viable pollen ratio of the transgenic rice is significantly higher compared with ZH11 control after drought stress during the flowering stage (Fig. 2, E and F). Whether the 10% higher viable pollen ratio would have a significant impact on seed setting is uncertain. Nevertheless, a higher viable pollen ratio would certainly be a beneficial factor for high seed setting under stress conditions. The mechanism for the increased pollen viability awaits further investigation, although it might apparently benefit from the enhanced oxidative stress tolerance.

Fourth, *AtEDT1/HDG11* overexpression plants have a well-developed root system (Fig. 6). A well-developed

root system is essential for plant to maximize water and nutrient uptake and thus is critical for increasing yield under soil-related stress (Serraj et al., 2009).

At last, the photosynthetic leaf area and photosynthetic rate of flag leaf are higher in *AtEDT1/HDG11* transgenic rice than that of the ZH11 control (Figs. 4 and 5). More than 90% of crop biomass is derived from photosynthetic products (Makino, 2011). The photosynthetic productivity depends mainly on photosynthetic leaf area, photosynthetic rate, and accumulative hours of photosynthesis (Chen et al., 2007). Flag leaf is thought to make the greatest contribution to grain filling compared with the other leaves of the same plant (Mahmood et al., 1991; Chen et al., 2007). It is reasonable to predict that grain yield can be substantially improved if the photosynthetic capacity of the flag leaves is raised. The *AtEDT1/HDG11* transgenic rice has larger flag leaf area (Fig. 4, C and D) with significantly increased photosynthetic rate, which will produce more net photosynthate and thus increase the grain yield. Furthermore, WUE of the *AtEDT1/HDG11* transgenic rice was also improved. Productivity in crop plants may be increased by improving WUE (Ehleringer et al., 1993).

Profound Impact of *AtEDT1/HDG11* Overexpression on Global Gene Expression in Transgenic Rice Is Consistent with the Observed Phenotypes

Transcriptomic comparisons could facilitate the identification of key genes and regulatory mechanisms for the drought tolerance. In this study, we compared the gene expression profiling of rice flag leaf between the transgenic line 2-56 and ZH11 control under normal condition and drought condition to identify DEGs . The DEGs between 2-56D and ZH11N did not significantly overlap with DEGs between ZH11D and ZH11N. However, many stress-related marker genes were detected upregulated in 2-56D as well as in ZH11D, with a more significant change in 2-56D. These results suggest that *AtEDT1/HDG11* can regulate a large set of genes different from that of ZH11 under both normal and drought stress conditions (Fig. 8; Table III). Meanwhile, it can also enhance the expression of many known stress-responsive genes (Table IV), which may make the plant more responsive to stress signaling.

Through detailed analysis of DEGs between 2-56N and ZH11N, we found that photosynthesis-related, carbon fixation-related, and oxidation reduction-related genes were significantly enriched in transgenic plant (Supplemental Tables S1 and S2). In addition, genes involved in regulation of transcription in transgenic plant were also enriched after drought treatment (Supplemental Table S3). It was reported that DEGs between superhybrid rice and its parents were found significantly enriched in pathways such as photosynthesis and carbon fixation (Bao et al., 2005; Wei et al., 2009; Song et al., 2010), providing another view for understanding the molecular mechanism underlying heterosis in rice. Interestingly, our results show a similar expression pattern in the *AtEDT1/HDG11* transgenic rice, which can partially explain the increased photosynthetic rate and grain yield in the transgenic rice.

Taken together, our gene expression profiling comparison results show a nice correlation of gene expression profile with the observed phenotypes of the transgenic rice regarding the enhanced photosynthesis and drought tolerance. However, DEGs of key developmental genes related to the observed phenotypes in root and inflorescence were not found in our DGE data because the material we used for DGE profiling was mature flag leaves at preanthesis stage. Thus, we did not detect DEGs involved in inflorescence or root development. We believe that overexpression of *AtEDT1/HDG11* in rice will change the expression profile of some development-related genes as in Arabidopsis *edt1* mutant (Yu et al., 2008). Future transcriptome analysis of root and reproductive organ should reveal the DEGs of the transgenic rice, which may help identify key genes involved in the improved root system and yield components.

MATERIALS AND METHODS

Construction and Transformation of *AtEDT1/HDG11*

The full-length complementary DNA sequence of *AtEDT1/HDG11* was isolated from the Columbia ecotype of Arabidopsis (*Arabidopsis thaliana*) using RT-PCR. The resulting amplified fragment was cloned into pCB2006 (Lei et al., 2007; Supplemental Fig. S1A). The construct was transformed into the rice (*Oryza sativa japonica*) ZH11 by the *Agrobacterium tumefaciens*-mediated transformation method (Hiei et al., 1994).

Identification of Transgenic Plants

Genomic DNA was isolated from the putative transgenic and wild-type rice. The PCR was used to screen for putative transgenic plants with the following primers: *bar*-LP (5′-TCAAATCTCGGTGACGGGCA-3′) and *bar*-RP (5′-GTCTGCACCATCGTCAACCACTA-3′). Amplified fragments were separated on 1% (w/v) agarose gel.

RT-PCR Analysis and Southern-Blot Analysis

Total RNA was extracted from both transgenic and wild-type seedlings using TRIzol reagent (Invitrogen), and 1 μg of total RNA from each sample was used for the reverse transcription reaction. The expression of *AtEDT1/HDG11* and *tubulin* (an internal standard) were analyzed using RT-PCR with the following primers: *tubulin*-LP (5′-GGAGATCCTCCACATCCAG-3′), *tubulin*-RP (5′-CAGAAAGGGTAGCATTGTAAG-3′), *AtEDT1/HDG11*-LP (5′-AGTGATTTCTTCAGGATGGGA-3′), and *AtEDT1/HDG11*-RP (5′-CGTTTGGTTCAGGGCTCTTA-3′).

Ten micrograms of genomic DNA digested with *Sac*I was used for Southern-blot analysis with a ^{32}P-dCTP-labeled *bar*-specific probe using standard protocols (Sambrook et al., 1989).

Drought Treatment, Physiological Characterization, and Grain Yield Analysis

For drought tolerance tests of plant at seedling stage, 2-week-old seedlings were transplanted to the soil and grown under standard growth conditions (14-h-light/10-h-dark cycle at 28°C), and then the plants were subjected to progressive drought conditions by withholding water for 6 to 9 d before rewatering. The entire test was repeated a minimum of three times. Leaves of similar developmental stages before and after drought treatment were used for measurement of soluble sugar content, Pro content, and SOD activity. Soluble sugars were determined spectrophotometrically by anthrone reagent using Glc as standard (Dubois et al., 1956). Pro was assayed using colorimetric

method (Bates et al., 1973). SOD activity was determined according to the method previously described (Hodges and Forney, 2000).

To test drought tolerance of plant at reproductive stage, drought treatments were applied at preanthesis stage (end of booting stage toward panicle emerging) by withholding water for 5 to 10 d followed by rewatering. Plants were rewatered when visual stress symptoms (e.g. leaf rolling) appeared in the transgenic plants. During the mature stage, yield and yield components data were collected.

To evaluate the water loss rate of the plant, flag leaf were detached from plant and weighed at different time intervals at RT. The proportion of fresh weight lost was calculated based on the initial weight of the leaf.

Field Trials

To evaluate yield and yield components of transgenic plants under normal and drought conditions in the field, two independent homozygous lines (2-56 and 14-16), together with ZH11 control, were transplanted to the fields of Sanya or Lingshui, Hainan Province, China, ideal places for rice drought tolerance testing because of the absence of rainfall from November to the following April. Rice seeds were germinated and transplanted as usual. One month after transplanting, when seedlings were established, water in the rice paddy field was discharged through the outlets surrounding the field, and no irrigation was applied through the rest of the growing season. The field trial consists of three replica plots of about 26 m^2 each. A duplicate set of materials was planted in another isolated field with full irrigation to evaluate the difference of yield between the transgenic and control rice under normal conditions. Yield and yield components data were collected from three 1-m^2 areas in each replica plot for statistical analysis.

Pollen Viability Analysis

Pollen grains from the transgenic plants and control plants were collected from spikelets just before flowering and stained with a 1% iodine-potassium iodide solution to observe starch accumulation (Jefferies, 1977). Stained pollen grains were examined directly under a microscope and photographed. Round, filled, and deep-color stained pollen was counted as fertile.

ABA Measurement

ABA measurements were conducted by the ABA immunoassay kit as described (Yang et al., 2001). Briefly, before drought treatment, 0.2 g of the 14-d-old seedlings of the *AtEDT1/HDG11* transgenic rice and the wild type grown on soil were used for ABA quantification. For drought treatment, watering was withheld for 4 d when visual stress symptoms appeared. Then, 0.2 g of seedlings was used for ABA quantification.

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

To measure stomatal density, leaves of the same age and from the same relative position were sampled from plants of the wild type and transgenic plants grown under the same conditions. A leaf surface imprint method was used as described (Yu et al., 2008). For statistical analysis of stomatal density, three leaves were sampled for each plant, and 10 plants were sampled for the wild type and the transgenic plants, respectively.

Photosynthesis (P) and transpiration (T) rates were measured using a portable photosynthesis system (Li-Cor LI-6400XT) in the morning (9 to 11 AM) on the same plants mentioned above before stomata observation. All of the photosynthetic measurements were taken at a constant air flow rate of 500 μmol s^{-1}. The concentration of CO_2 was 400 μmol mol^{-1} using the system's CO_2 injector (Li-Cor 6400-01), and the temperature was maintained at 26 ± 2°C, and the photosynthetic photon flux density was 1,200 μmol (photon) m^{-2} s^{-1}. Three measurements were made for each plant, and 10 plants were used for both the wild-type and the transgenic plants. WUE was defined as P/T ratio and derived from the measured P and T.

Morphological Characterization of Roots

Seeds were soaked in water at room temperature for 2 d and then germinated on wet filter paper at 37°C for 5 d. The most uniformly germinated seeds were sown in wet vermiculite in 10- × 25-cm pots. Five days later, the seedlings were cultured with Yoshida's culture solution. The seedlings were allowed to grow in the greenhouse for the indicated days until the whole plants were pulled out carefully, and the vermiculite was washed away carefully to collect roots. Root number was counted and the longest root length and root biomass were measured.

DGE Analysis

AtEDT1/HDG11 transgenic rice and ZH11 rice at preanthesis stage in the greenhouse were withheld water for 5 to 6 d until leaves were half rolled and leaf relative water content was around 80%. Flag leave at the same age were harvested, snap frozen immediately in nitrogen, and stored at –80°C until further processing. Three independent replicates were collected (each from individual plant). Samples from normal grown plants corresponding to the drought treatment were also collected at the same time as respective controls.

Total RNAs were extracted from the samples using TRIzol reagent (Invitrogen) and treated with DNase I (Fermentas) according to the manufacturer's instructions. RNA quality and purity were assessed with optical density at 260 nm/optical density at 280 nm. RNAs from three independent replicates were mixed by equal volume. Twenty micrograms of total RNA were used for Illumina DGE tag profiling processed by BioMarker Technologies. Sequence tag preparation was performed with the Illumina DGE tag profiling kit according to the manufacturer's protocol. More than 5.2 million clean tags were obtained in each sample. All clean tags were mapped to the rice reference sequence, and no more than one nucleotide mismatch was allowed. The clean tags mapped to reference sequences from multiple genes were filtered. The remaining clean tags were designed as perfect clean tags. The number of perfect clean tags for each gene was calculated and then normalized in reads per kilobase of exon model per million mapped reads (RPKM) using the method described by Mortazavi et al., (2008). DEGs were defined by using IDEG6 (Romualdi et al., 2003), with a relative change threshold of 2-fold ($P < 0.005$, false discovery rate < 0.01). For hierarchical clustering analysis, the software Cluster 2.20 was used. Functional annotation analysis of DEGs was performed by the DAVID (Huang et al., 2008) Web tools.

Quantitative RT-PCR Validation

We selected nine functionally important and representative DEGs for validation using quantitative RT-PCR. Gene-specific primers were designed for each DEG (Supplemental Table S4), and the rice *β-actin* gene was used as a control. Residual RNA samples for DGE analysis were subjected to quantitative RT-PCR analysis. Quantitative RT-PCR was performed by using a TaKaRa SYBR Premix Ex Taq II reagent kit. The results were based on the average of three parallel experiments.

Statistical Analysis

The ANOVA was used to compute statistically significant differences ($P < 0.05$, $P < 0.01$, or $P < 0.001$) based on the Student's t test. Data are the means ± SD of three independent replicates.

Supplemental Data

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

Supplemental Figure S1. Generation of transgenic plants overexpressing *AtEDT1/HDG11*.

Supplemental Figure S2. Reduced leaf stomatal density and enlarged stomatal size of the flag leaf at flowering stage.

Supplemental Figure S3. Quantitative real-time PCR validation of the results of DGE tag profiling.

Supplemental Table S1. Biological process categories involved in photosynthesis, oxidation-reduction are significantly enriched in DEGs between 2-56N and ZH11N.

Supplemental Table S2. Clusters of photosynthesis-related genes have higher enrichment score in DEGs between 2-56N and ZH11N.

Supplemental Table S3. Genes related to photorespiration, carbon fixation, regulation of transcription and oxidation-reduction are significantly enriched in DEGs between 2-56D and ZH11D.

Supplemental Table S4. Primers used for quantitative RT-PCR.

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