Root-Specific Expression of OsNAC10 Improves Drought Tolerance and Grain Yield in Rice under Field Drought Conditions\(^1\text{[W]}\text{[OA]}\)

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Drought poses a serious threat to the sustainability of rice (Oryza sativa) yields in rain-fed agriculture. Here, we report the results of a functional genomics approach that identified a rice NAC (an acronym for NAM [No Apical Meristem], ATAF1-2, and CUC2 [Cup-Shaped Cotyledon]) domain gene, OsNAC10, which improved performance of transgenic rice plants under field drought conditions. Of the 140 OsNAC genes predicted in rice, 18 were identified to be induced by stress conditions. Phylogenetic analysis of the 18 OsNAC genes revealed the presence of three subgroups with distinct signature motifs. A group of OsNAC genes were prescreened for enhanced stress tolerance when overexpressed in rice. OsNAC10, one of the effective members selected from prescreening, is expressed predominantly in roots and panicles and induced by drought, high salinity, and abscisic acid. Overexpression of OsNAC10 in rice under the control of the constitutive promoter GOS2 and the root-specific promoter RC3 increased the plant tolerance to drought, high salinity, and low temperature at the vegetative stage. More importantly, the RC3: OsNAC10 plants showed significantly enhanced drought tolerance at the reproductive stage, increasing grain yield by 25% to 42% and by 5% to 14% over controls in the field under drought and normal conditions, respectively. Grain yield of GOS2: OsNAC10 plants in the field, in contrast, remained similar to that of controls under both normal and drought conditions. These differences in performance under field drought conditions reflect the differences in expression of OsNAC10-dependent target genes in roots as well as in leaves of the two transgenic plants, as revealed by microarray analyses. Root diameter of the RC3: OsNAC10 plants was thicker by 1.25-fold than that of the GOS2:OsNAC10 and nontransgenic plants due to the enlarged stele, cortex, and epidermis. Overall, our results demonstrated that root-specific overexpression of OsNAC10 enlarges roots, enhancing drought tolerance of transgenic plants, which increases grain yield significantly under field drought conditions.

Plants respond and adapt to abiotic stresses to survive under adverse conditions. Upon exposure of plants to such stresses, many genes are induced, and their products are involved in the protection of cellular machinery from stress-induced damage (Bray, 1993; Thomashow, 1999; Shinozaki et al., 2003). The expression of stress-related genes is largely regulated by specific transcription factors. The overexpression of such transcription factor genes often results in activation of many functional genes related to the particular stress conditions, consequently conferring stress tolerance. For example, the DREB1A/CFB3 gene in transgenic Arabidopsis (Arabidopsis thaliana) activates expression of its stress-related downstream genes, thereby enhancing stress tolerance (Liu et al., 1998; Kasuga et al., 1999).

The rice (Oryza sativa) and Arabidopsis genomes each encode more than 1,300 transcriptional regulators, which account for 6% of the estimated total number of genes in each plant. About 45% of these transcription factors are reported to be from gene families specific to plants (Riechmann et al., 2000; Kikuchi et al., 2003). One example of such a plant-specific family of transcription factors is the NACs. The NAC acronym is derived from the names of the three genes that were first described as containing a NAC domain, namely NAM (for No Apical Meristem), ATAF1-2, and CUC2 (for Cup-Shaped Cotyledon). The NAC domain is a highly conserved N-terminal DNA-binding domain, and NAC proteins also contain a variable C-terminal domain (Ooka et al., 2003; Jeong et al., 2009) and appear to be widespread in plants. The genomes of rice and Arabidopsis were initially predicted to contain 105 and 75 NAC genes, respectively.
(Ooka et al., 2003; Xiong et al., 2005). Later, 140 NAC genes were identified in rice (Fang et al., 2008). However, only a few of these genes have been characterized so far, and these show diverse functions in both plant development and stress responses. The earliest reported NAC genes include NAM from petunia (Petunia hybrida) that determines the position of the shoot apical meristem (Sour et al., 1996) and CUC2 from Arabidopsis that participates in the development of embryos and flowers (Aida et al., 1997). In addition, the Arabidopsis NAP gene regulates cell division and cell expansion in flower organs (Sablowski and Meyerowitz, 1998) and the AtNAC1 gene mediates auxin signaling to promote lateral root development (Xie et al., 2000). Genes in the ATAF subfamily, including SNAC (Collinge and Boller, 2001) from potato (Solanum tuberosum) and ATAF1-2 (Aida et al., 1997) from Arabidopsis, are induced by pathogen attack and wounding. More recently, AtNAC072 (RD29), AtNAC019, AtNAC019, and AtNAC012 from Arabidopsis (Fujita et al., 2004; Tran et al., 2004; Christianson et al., 2009), BnNAC from Brassica napus (Hegedus et al., 2003), and SNAC1 and SNAC2 from rice (Hu et al., 2006, 2008) have been found to be involved in responses to various environmental stresses. AtNAC2, another stress-related NAC gene in Arabidopsis, functions downstream of the ethylene and auxin signal pathways and enhances salt tolerance when overexpressed (He et al., 2005). A wheat (Triticum aestivum) NAC gene, NAM-B1, has been reported to be involved in nutrient remobilization from leaves to developing grains (Uaury et al., 2006).

Drought is one of the major constraints to rice production worldwide. In particular, exposure to drought conditions during the panicle development stage results in a delayed flowering time, reduced number of spikelets, and poor grain filling. To date, a number of studies have suggested that the overexpression of stress-related genes may improve drought tolerance in rice to some extent (Xu et al., 1996; Garg et al., 2002; Jang et al., 2003; Hu et al., 2006, 2008; Ito et al., 2006; Nakashima et al., 2007; Oh et al., 2007). However, despite a number of such efforts to develop drought-tolerant rice plants, very few have shown an improvement in grain yields under field conditions. These include transgenic rice plants expressing SNAC1 (Hu et al., 2006), OsLEA3 (Xiao et al., 2007), and AP37 (Oh et al., 2009).

In this study, a genome-wide analysis of rice NAC transcription factors was conducted to identify genes that improve tolerance to environmental stress. A group of OsNAC genes were prescreened for enhanced stress tolerance when overexpressed in rice. Here, we report the role of OsNAC10, one of the effective members selected from the prescreening, in drought tolerance. Overexpression of OsNAC10 under the control of GOS2 (de Pater et al., 1992), a constitutive promoter, and RCC3 (Xu et al., 1995), a root-specific promoter, improved plant tolerance of transgenic rice to drought, high salinity, and low temperature during the vegetative stage of growth. In addition, RCC3:OsNAC10 plants showed significantly enhanced drought tolerance at the reproductive stage, with a grain yield increase of 25% to 42% over the controls under field drought conditions. Grain yield of GOS2:OsNAC10 plants under the same conditions, in contrast, remained relatively unchanged, demonstrating the potential use of the root-specific expression strategy for improving drought tolerance in rice.

RESULTS

Identification of Stress-Inducible Rice NAC Domain Genes

Previously, the rice genome was predicted to contain 140 OsNAC genes (Fang et al., 2008). To identify those that are stress inducible, we performed expression profiling with the Rice 3’Tiling microarray (GreenGene Biotech). We obtained RNAs from the leaves of 14-d-old rice seedlings that had been subjected to drought, high salinity, abscisic acid (ABA), and low temperature. When three replicates were averaged and compared with untreated leaves, a total of 18 OsNAC genes were found to be up-regulated by 1.9-fold or greater (P < 0.05) upon exposure to one or more stress conditions (Table I). Phylogenetic analysis of the amino acid sequences of the corresponding 18 OsNAC proteins revealed the presence of three subgroups (I–III; Fig. 1; Supplemental Fig. S1). Furthermore, comparison of the amino acid sequences spanning the NAC domains in these proteins revealed signature motifs by which these subgroups can be distinguished (Fig. 1). For example, signature motifs Ia-c and Ia-c are specific to subgroups I and II, respectively. In addition to sequence similarities, the members of each subgroup were found to be closely related in terms of their response to stress. For example, the expression of the genes in subgroups II and III is not induced by low temperature.

One of the stress-responsive OsNAC genes, OsNAC10 (AK069257), which is in subgroup I, was functionally characterized in this study. Reverse transcription (RT)-PCR analysis of this gene in various tissues at different stages of development revealed that it is predominantly expressed in roots and flowers (panicles; Fig. 2A). We also performed RNA gel-blot analysis using total RNAs from leaf tissues of 14-d-old seedlings exposed to high salinity, drought, ABA, and low temperature (Fig. 2B). Consistent with the results from our microarray experiments, the expression of OsNAC10 was induced by drought, high salinity, and ABA but not by low temperature.

Stress Tolerance of RCC3:OsNAC10 and GOS2:OsNAC10 Plants at the Vegetative Stage

To enable the overexpression of the OsNAC10 genes in rice, their full-length cDNAs were isolated and linked to the RCC3 promoter (Xu et al., 1995) for root-specific expression and to the GOS2 promoter (de
Overexpressed \( \text{OsNAC10} \) and \( \text{GOS2:OsNAC10} \) plants. The transgenic plants also recovered more quickly than the NT plants upon rewatering. Consequently, the NT plants remained severely affected by drought stress at the time at which some of the transgenic lines had partially recovered (Fig. 3B). To further verify this stress tolerance phenotype, we measured the \( F_v/F_m \) values of the transgenic and NT control plants, all at the vegetative stage (Fig. 3C). These values represent the maximum photochemical efficiency of PSI during the dark-adapted state \( (F_v, \text{variable fluorescence}) \) and were found to be 15% to 30% higher in the \( \text{Rc3:OsNAC10} \) and \( \text{GOS2:OsNAC10} \) plants compared with the NT plants under drought and low-temperature conditions. Under moderate salinity conditions, the \( F_v/F_m \) levels were also higher in both transgenic plants by 10% compared with the NT controls. In contrast, under severe salinity conditions, these levels were similar in all plant types. Our results thus indicate that the overexpression of \( \text{OsNAC10} \) in transgenic rice primarily increases their tolerance to drought and low-temperature stress conditions during the vegetative stage of growth.

### Identification of Genes Up-Regulated by Overexpressed \( \text{OsNAC10} \)

To identify genes that are up-regulated by the overexpression of \( \text{OsNAC10} \), we performed expression

<table>
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<th>Subgroup</th>
<th>Sequence ID</th>
<th>High Salinity</th>
<th>Drought</th>
<th>ABA</th>
<th>Low Temperature</th>
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<tr>
<td>I</td>
<td>( \text{OsNAC10} )</td>
<td>16.22 0.000 14.91 0.000</td>
<td>13.81 0.000 0.88 0.788</td>
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<td></td>
<td>( \text{SNAC1} )</td>
<td>17.31 0.000 17.18 0.000</td>
<td>1.90 0.013 6.96 0.000</td>
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<td></td>
<td>( \text{OsNAC6} )</td>
<td>5.94 0.000 5.16 0.000</td>
<td>4.62 0.000 2.80 0.011</td>
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<td></td>
</tr>
<tr>
<td></td>
<td>( \text{OsNAC5} )</td>
<td>6.74 0.000 7.10 0.000</td>
<td>1.39 0.190 0.72 0.268</td>
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<td>11.26 0.000 1.14 0.709</td>
<td>6.00 0.001 0.69 0.415</td>
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<td>( \text{Os01g0816100} )</td>
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<td>2.23 0.041 5.35 0.007</td>
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<td>( \text{Os12g0132700} )</td>
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\( a \)Sequence identification numbers for the full-length cDNA sequences of the corresponding genes.

\( b \)The mean of three independent biological replicates. These microarray data sets can be found at http://www.ncbi.nlm.nih.gov/geo/ (Gene Expression Omnibus).

\( c \)P values were analyzed by one-way ANOVA.
profiling of the GOS2:OsNAC10 and RCC3:OsNAC10 plants in comparison with NT controls under normal growth conditions. This profiling was conducted using the Rice 3'-Tiling microarray with RNA samples extracted from 14-d-old roots and leaves of each plant, all grown under normal growth conditions. Each data set was obtained from three biological replicates. As listed in Table II and Supplemental Tables S1 and S2, statistical analysis of each data set using one-way ANOVA identified a total of 34 root-specific and 40 leaf-specific target genes that are up-regulated following OsNAC10 overexpression with a 3-fold or greater induction in the transgenic plants compared with NT plants (P, 0.05). More specifically, up-regulation of 34 genes was specific to roots of both RCC3:OsNAC10 and GOS2:OsNAC10 plants, whereas up-regulation of 40 genes was specific to leaves of the GOS2:OsNAC10 plants. Surprisingly, only four genes were commonly activated in both roots and leaves of the GOS2:OsNAC10 plants, indicating that OsNAC10 activates different sets of target genes in different tissues. We selected 11 target genes (seven root specific, two leaf specific, and two common) and verified their OsNAC10-dependent expression patterns in roots and leaves of transgenic plants under normal growth conditions by real-time PCR (Fig. 4; Supplemental Fig. S2). The OsNAC10 mRNA levels were higher in roots and leaves of GOS2:OsNAC10 plants but only in the roots of RCC3:OsNAC10 plants compared with the respective NT controls. Transcript levels of seven root-specific target genes [P450, Zn-finger, HAK5, 2OG-Fe(II), NCED, NAC, and Klip3] were increased in transgenic roots as compared with NT roots yet remained similar in leaves of all plant types, verifying their OsNAC10-dependent expression in roots. Conversely, expression of two leaf-specific target genes, LRR and Peroxidase, was found to be OsNAC10 dependent only in GOS2:OsNAC10 leaves but not in RCC3:OsNAC10 leaves. Expression of two common target genes, F-box and MutS4, was found to be OsNAC10 dependent both in roots and leaves of the GOS2: OsNAC10 plants. We also measured transcript levels of the 11 target genes in roots and leaves of RCC3:OsNAC10, GOS2:OsNAC10, and NT plants after exposure to drought, high salinity, and low-temperature conditions (Fig. 4; Supplemental Fig. S2). Expression of the genes was further increased at various levels by stress treatments. Taken together, our results suggest that both the constitutive and root-specific overexpression of OsNAC10 enhances the stress tolerance of transgenic plants during the vegetative growth by activating different groups of target genes in different tissues.

**Root-Specific Overexpression of OsNAC10 Increases Rice Grain Yield under Field Drought Conditions**

A phenotypic evaluation of RCC3:OsNAC10, GOS2:OsNAC10, and NT control plants revealed no major differences at the vegetative growth stage of the entire

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**Figure 1.** Alignments of NAC domain sequences from 18 stress-inducible rice genes. The deduced amino acid sequences of the NAC domains from these 18 genes (Table I) were aligned using the ClustalW program. Identical and conserved residues are highlighted (gray). Signature motifs are indicated by the boxes: Ia to Ic and IIa to IIc for subgroups I and II, respectively.
plants. We evaluated yield components of the transgenic plants under normal and field drought conditions for two cultivating seasons (2008 and 2009). Three independent T4 (2008) and T5 (2009) homozygous lines of each of the
RCc3:OsNAC10 and GOS2:OsNAC10 plants, together with NT controls, were transplanted to a paddy field and grown to maturity. Yield parameters were scored for 20 (2008) and 30 (2009) plants per transgenic line from two (2008) and three (2009) replicates. Two data sets are generally consistent, with the 2009 data exhibiting greater statistical rigor. The grain yield of the GOS2:OsNAC10 plants remained similar to that of the NT controls under normal field conditions in both seasons (Fig. 5A; Supplemental Table S3). Filling rate and 1,000 grain weight of the GOS2:OsNAC10 plants were markedly reduced, and the reduction appeared to be balanced by the increase in numbers of panicles and total spikelets, consequently maintaining similar levels of total grain weight to that of the NT controls. In the RCc3:OsNAC10 plants under the same field conditions, however, total grain weight was increased by 5% to 14% compared with the NT controls, which was due to increased numbers of filled grains and total spikelets. These observations prompted us to examine the yield components of the transgenic rice plants grown under field drought conditions. Three independent T4 (2008) and T5 (2009) lines of the RCc3:OsNAC10, GOS2:OsNAC10, and NT plants were transplanted to a paddy field with a removable rain-off shelter and exposed to drought stress at the panicle heading stage (from 10 d before heading to 20 d after heading). The level of drought stress imposed under the rain-off shelter was equivalent to those that give 40% to 50% of total grain weight obtained under normal growth conditions, which was evidenced by the difference in

Figure 2. Expression of OsNAC10 in rice under different stress conditions and in various tissues at different developmental stages. A, Rice seeds were germinated and grown on MS agar medium in the dark for 2 d (D2) and then in the light for 1 d at 28°C (L1). The seedlings were transplanted into soil pots and grown in the greenhouse for 14 d (14d), until meiosis (M), until just before heading (BH), and until right after heading (AH). L1C, Coleoptiles from L1 seedlings. RT-PCR analyses were performed using RNAs from the indicated tissues at the indicated stages of development and gene-specific primers. The expression levels of a rice ubiquitin (OsUbi) were used as an internal control. B, Ten micrograms of total RNA was prepared from the leaf and root tissues of 14-d-old seedlings exposed to drought, high salinity, ABA, or low temperature for the indicated time periods. For drought stress, the seedlings were air dried at 28°C; for high-salinity stress, seedlings were exposed to 400 mM NaCl at 28°C; for low-temperature stress, seedlings were exposed to 4°C; for ABA treatment, seedlings were exposed to a solution containing 100 μM ABA. Total RNAs were blotted and hybridized with OsNAC10 gene-specific probes. The blots were then reprobed with the Dip1 gene, which was used as a marker for the up-regulation of key genes following stress treatments. Ethidium bromide (EtBr) staining was used to determine equal loading of RNAs.
levels of total grain weight of NT plants between the normal and drought conditions (Supplemental Tables S3 and S4). Statistical analysis of the yield parameters scored for two cultivating seasons showed that the decrease in grain yield under drought conditions was significantly smaller in the \textit{RCc3:OsNAC10} plants than that observed in the NT controls. Specifically, in the drought-treated \textit{RCc3:OsNAC10} plants, the number of filled grains was 26\% to 47\% higher than the drought-treated NT plants, which resulted in a 25\% to 42\% increase in the total grain weight, depending on transgenic line (Fig. 5; Supplemental Table S4). In the drought-treated \textit{GOS2:OsNAC10} plants, in contrast, the total grain weight remained similar to the drought-treated NT controls. Given the similar levels of drought tolerance during the vegetative stage in the \textit{RCc3:OsNAC10} and \textit{GOS2:OsNAC10} plants, these differences in grain yield under field drought conditions are surprising. Unlike in \textit{RCc3:OsNAC10} plants, spikelet development in \textit{GOS2:OsNAC10} plants is significantly affected by the constitutive overexpression of OsNAC10 under both normal and drought conditions.

We have measured root volume, length, dry weight, and diameter of \textit{RCc3:OsNAC10}, \textit{GOS2:OsNAC10}, and NT plants after growth to the heading stage of reproduction (Fig. 6). As shown in Figure 6, root diameter of the \textit{RCc3:OsNAC10} plants was thicker by 1.25-fold than that of the \textit{GOS2:OsNAC10} and NT plants. Microscopic analysis of cross-sectioned roots revealed that the increase in root diameter was due to the enlarged stele, cortex, and epidermis of \textit{RCc3:OsNAC10} roots. These observations suggest that root-specific overexpression of \textit{OsNAC10} enlarges roots, enhancing drought tolerance of transgenic plants at the reproductive stage.

**DISCUSSION**

In this study, we performed expression profiling using RNAs from stress-treated rice plants and identified 18 NAC domain factors that are stress inducible (Table I). Alignment of these stress-inducible factors further revealed three subgroups, within which the members are more closely related, suggesting a common stress response function. Overexpression of one such gene, \textit{OsNAC10}, under the control of the constitutive promoter \textit{GOS2} (\textit{GOS2:OsNAC10}) and the root-
specific promoter RCc3 (RCc3:OsNAC10) was found to increase rice plant tolerance to drought and low temperature at the vegetative stage of growth. Increased tolerance to a moderate level of salinity conditions was also observed in both of these transgenic plants. More importantly, the RCc3:OsNAC10 plants showed significantly enhanced drought tolerance at the reproductive stage, increasing grain yield by 25% to 42% and by 5% to 14% over controls in the field under drought and normal conditions, respectively. These results were consistent in our evaluations for two consecutive years (2008 and 2009). In contrast, grain yield in GOS2:OsNAC10 plants in the field remained similar to that of controls under both normal and drought conditions. In GOS2:OsNAC10 plants, the increased expression of OsNAC10 in the whole plant body, including floral organs, resulted in a large reduction in filling rate under both normal and drought conditions. In particular, the large decrease in filling rate of GOS2:OsNAC10 plants under drought conditions did not
Figure 4. Regulated genes in roots and leaves of Rcc3: OsNAC10 and GOS2:OsNAC10 plants under normal and stress conditions. The transcript levels of OsNAC10 and six target genes were determined by quantitative RT-PCR (using the primers listed in Supplemental Table S5), and those in Rcc3:OsNAC10 and GOS2:OsNAC10 transgenic rice plants are presented as relative to the levels in untreated NT control roots and leaves, respectively. Data were normalized using the rice ubiquitin gene (OsUbi) transcript levels. Values are means ± sd of three independent experiments.
allow the total grain weight to increase, even with the significant increase in numbers of panicles, and spikelets per panicle, over the controls. The RCc3:OsNAC10 plants, in contrast, were effective against drought stress at both the reproductive and vegetative stages. The root-specific overexpression of OsNAC10 does not seem to affect the development of reproductive organs while conferring stress tolerance in the transgenic plants. The lower decrease in the filling rate as well as in the numbers of panicles and spikelets per panicle in RCc3:OsNAC10 plants than in NT controls under drought conditions is clear evidence of drought tolerance at the reproductive stage.

The vegetative growth of both the GOS2:OsNAC10 and RCc3:OsNAC10 plants was visually indistinguishable from the NT controls. Given the different numbers of target genes that are up-regulated in RCc3:OsNAC10 and GOS2:OsNAC10 roots and leaves, differences in their response to drought at the reproductive stage were not unexpected. Our microarray experiments identified 44 and 59 up-regulated root-expressed genes that are specific to the RCc3:OsNAC10 and GOS2:OsNAC10 plants, respectively, in addition to the 34 up-regulated root-expressed genes that are common to both plants. We also learned that OsNAC10-dependent target genes identified in roots are very different from those in leaves of the GOS2:OsNAC10 plants. Only four out of 40 target genes are common to both leaves and roots of the GOS2:OsNAC10 plants. Our real-time PCR experiments verified their OsNAC10-dependent up-regulation in roots and/or leaves of RCc3:OsNAC10 and GOS2:OsNAC10 plants. In addition, expression patterns and levels of the target genes were very different in roots and leaves of RCc3:OsNAC10 and GOS2:OsNAC10 plants under both unstressed and stressed conditions (Fig. 4; Supplemental Fig. S2). These observations led us to conclude that OsNAC10 regulates different sets of target genes in different tissues at various stages of plant growth. And the difference in drought tolerance between RCc3:OsNAC10 and GOS2:OsNAC10 plants at the reproductive stage reflects the difference in expression of target genes in leaves as well as in roots. One is often aware of discrepancies in lists of target genes identified in transgenic rice with the same transgene. For example, none of the target genes identified in transgenic rice with Ubi:OsNAC6 (or SNAC2; Nakashima et al., 2007) is overlapped with those identified in transgenic rice with Ubi:SNAC2 (or OsNAC6; Hu et al., 2008). The microarray experiments were performed using 14-d-old seedlings for the former and four-leaf stage leaf tissues for the latter. Similarly, two research groups (Oh et al., 2005; Ito et al., 2006) have reported very different lists of target genes identified in transgenic rice plants with the same transgene, Ubi:CBF3/DREB1A. Thus, it is not surprising to see the differences if the target genes analyzed were in different tissues and/or stages of transgenic plants. We do not rule out the possibility that such discrepancies may have been in part due to the different genetic backgrounds of rice cultivars.

Figure 5. Agronomic traits of RCc3:OsNAC10 and GOS2:OsNAC10 plants grown in the field under both normal and drought conditions. Spider plots of the agronomic traits of three independent homozygous T4 and T5 lines of RCc3:OsNAC10 and GOS2:OsNAC10 plants and corresponding NT controls under both normal and drought conditions were drawn using Microsoft Excel. Each data point represents the percentage of the mean values (n = 20 and n = 30) listed in Supplementary Tables S3 and S4. The mean measurements from the NT controls were assigned a 100% reference value. CL, Culm length; PL, panicle length; NP, number of panicles per hill; NSP, number of spikelets per panicle; TNS, total number of spikelets; FR, filling rate; NFG, number of filled grains; TGW, total grain weight; 1,000GW, 1,000 grain weight.
The target genes identified in both the RCc3:OsNAC10 and GOS2:OsNAC10 roots include genes that function in stress responses, such as Cytochrome P450, NCED, and Potassium transporter HAK5. Also included are seven protein kinases and five transcription factors containing domains such as AP2, WRKY, LRR, NAC, and Zn-finger that also function in stress tolerance pathways. We further identified target genes encoding proteins that function as osmolytes, such as Potassium transporter KUP3 and Heavy metal transporter, and that function in reactive oxygen species-scavenging systems, such as Multicopper oxidase, Detoxification protein, Chitinase, and Glycosyl hydrolase. The expression of such target genes may enhance grain yield under both normal and drought conditions.

Recently, the SNAC1 gene, a member of our subgroup I (Table I), was shown to confer tolerance of transgenic rice plants to field drought conditions as well as to drought and high salinity at the vegetative stage (Hu et al., 2006). This is consistent with our results here for RCc3:OsNAC10 plants. In the case of low temperatures at the vegetative stage, however, the effect was more pronounced in our transgenic plants harboring OsNAC10. In addition, SNAC1 did not affect grain yield in transgenic plants grown under normal conditions, while a 5% to 14% increase in grain yield was observed in our RCc3:OsNAC10 plants in normal field conditions. Transgenic rice harboring OsNAC6 (or SNAC2), another member of subgroup I, was shown previously to display enhanced tolerance at the vegetative stage to cold, salt, and blast disease as a result of the increased expression of stress-related genes (Nakashima et al., 2007; Hu et al., 2008). Despite their high protein sequence homology (70%–73%) within the NAC domain, the SNAC1 and OsNAC6 (or SNAC2) genes are distinct from OsNAC10 in that their expression is increased upon exposure to low-temperature conditions (Table I), which may be responsible in part for the observed functional differences between these genes and OsNAC10.

To date, the potential impact of homeotic genes like the NAC factors upon grain yield have received relatively little attention due to their negative effects on fertility, plant growth, and development. Transgenic rice plants overexpressing OsNAC6 in a whole plant body exhibit growth retardation and low reproductive yield (Nakashima et al., 2007). In this study, we also observed a yield penalty under drought conditions when the OsNAC10 overexpressed in a whole body in
the GOS2:OsNAC10 plants; in RCC3:OsNAC10 plants, however, significant increase in grain yield was observed. Interestingly, the targeted expression of the prokaryotic Na+/H+ antiporter gene in roots of transgenic tobacco (Nicotiana tabacum) plants has been shown to confer a higher tolerance to high-salinity conditions compared with whole plant expression (Hossain et al., 2006). More importantly, Na+ content in leaves of transgenic tobacco plants with root-specific expression of the Na+/H+ antiporter was lower than that of plants that constitutively expressed this gene, although there was no expression of this transgene in the leaf of the former. These observations together with our results suggest that ectopic expression of a stress response gene in a whole plant may not be as effective as root-specific expression on stress tolerance. This is particularly true for hemeogenic genes that function in the development of reproductive organs.

The Arabidopsis HARDY (HRD) gene, an AP2 transcription factor, has been previously found to provide enhanced drought tolerance in transgenic Arabidopsis and rice plants (Karaba et al., 2007). HRD was isolated by activation tagging in Arabidopsis; the activation-tagged line had a robust root system with increased numbers of secondary and tertiary roots. We have tagged line had a robust root system with increased by activation tagging in Arabidopsis; the activation- and rice plants (Karaba et al., 2007).

The Arabidopsis GOS2 (OsNAC10) gene, which encodes a transcription factor, has been previously found to provide enhanced drought tolerance in transgenic Arabidopsis (Hossain et al., 2006). More importantly, Na+ content in leaves of transgenic tobacco plants with root-specific expression of the Na+/H+ antiporter was lower than that of plants that constitutively expressed this gene, although there was no expression of this transgene in the leaf of the former. These observations together with our results suggest that ectopic expression of a stress response gene in a whole plant may not be as effective as root-specific expression on stress tolerance. This is particularly true for hemeogenic genes that function in the development of reproductive organs.

The Arabidopsis HARDY (HRD) gene, an AP2 transcription factor, has been previously found to provide enhanced drought tolerance in transgenic Arabidopsis and rice plants (Karaba et al., 2007). HRD was isolated by activation tagging in Arabidopsis; the activation-tagged line had a robust root system with increased numbers of secondary and tertiary roots. We have measured root volume, length, dry weight, and diameter of RCC3:OsNAC10, GOS2:OsNAC10, and NT plants after growth to the stage of reproduction (Fig. 6). Root diameter of the RCC3:OsNAC10 plants was thicker than that of the GOS2:OsNAC10 and NT plants. The increase in root diameter of the RCC3:OsNAC10 plants appears to be caused by an increase in cell number rather than cell size, as evidenced by the similar size of epidermal and exodermal cells between NT and RCC3:OsNAC10 roots. It was shown that vigorous growth of roots with an increase in length and thickness is correlated with drought tolerance and grain yield of rice (Ekanayake et al., 1985; Price et al., 1997). How the thicker roots of the RCC3:OsNAC10 plants are associated with higher grain yield remains to be investigated.

In summary, we report an analysis of the rice NAC domain family in their responses to stress treatments. More importantly, we evaluated agronomic traits in transgenic crops throughout the entire stages of plant growth in the field, which allowed us to address the advantages of using such a regulatory gene as OsNAC10 for improving stress tolerance. Finally, we demonstrated that a root-specific rather than whole body expression of OsNAC10 increases rice grain yield under drought conditions without yield penalty, providing the potential use of this strategy for improving drought tolerance in other crops.

**MATERIALS AND METHODS**

**Plasmid Construction and Transformation of Rice**

The coding region of OsNAC10 was amplified from rice (Oryza sativa) total RNA using an RT-PCR system (Promega) according to the manufacturer's instructions. Primer pairs were as follows: forward (5'-ATGCCGAG-CACGCCGGGCGC-3') and reverse (5'-CTACTCTCCTGACCATGATG-3'). To enable the overexpression of the OsNAC10 gene in rice, the cDNA for this gene was linked to the GOS2 promoter for constitutive expression and to the RCC3 promoter for root-specific expression using the Gateway system (Invitrogen). Plasmids were introduced into Agrobacterium tumefaciens LBA4404 by triparental mating, and embryogenic (cv Nipponbare) calli from mature seeds were transferred as described previously (Jang et al., 1999).

**Protein Sequence Analysis**

Of 140 NAC factors predicted from the rice genome (Fang et al., 2008), we selected 87 NAC protein sequences that have full-length EST information from a National Center for Biotechnology Information database search using the tBLASTN program and previously reported annotation of the NAC family (Ooka et al., 2003; Xiong et al., 2005). Amino acid sequences of 87 NAC domains were aligned using ClustalW followed by construction of a neighbor-joining phylogenetic tree using the MEGA program (8 = 1,000 bootstrap replications).

**Northern-Blot Analysis**

Rice (cv Nipponbare) seeds were germinated in soil and grown in a greenhouse (16-h-light/8-h-dark cycle) at 22°C. For high-salinity and ABA treatments, 14-d-old seedlings were transferred to nutrient solution containing 400 mM NaCl or 100 μM ABA for the indicated periods in the glasshouse under continuous light of approximately 1,000 μmol m⁻² s⁻¹. For drought treatment, 14-d-old seedlings were excised and air dried for the indicated time course under continuous light of approximately 1,000 μmol m⁻² s⁻¹. For low-temperature treatments, 14-d-old seedlings were exposed at 4°C in a cold chamber for the indicated time course under continuous light of 150 μmol m⁻² s⁻¹. The preparation of total RNA and RNA gel-blot analysis were performed as reported previously (Jang et al., 2002).

**Drought Treatments of Rice Plants at the Vegetative Stage**

Transgenic and NT rice (cv Nipponbare) seeds were germinated in half-strength Murashige and Skoog (MS) solid medium in a growth chamber in the dark at 28°C for 4 d, transplanted into soil, and then grown in a greenhouse (16-h-light/8-h-dark cycles) at 28°C to 30°C. Eighteen seedlings from each transgenic and NT line were grown in pots (3 × 3 × 5 cm; one plant per pot) for 4 weeks before undertaking the drought stress experiments. To induce drought stress, 4-week-old transgenic and NT seedlings were unwatered for 3 d followed by 2 d of watering. The numbers of plants that survived or continued to grow were then scored.

**Measurement of Chlorophyll Fluorescence under Drought, High-Salinity, and Low-Temperature Conditions**

Transgenic and NT rice (cv Nipponbare) seeds were germinated and grown in half-strength MS solid medium for 14 d in a growth chamber (16-h-light [150 μmol m⁻² s⁻¹]/8-h-dark cycle at 28°C). The green portions of approximately 10 seedlings were then cut using a scissors prior to stress treatments in vitro. All stress treatments were conducted under continuous light at 150 μmol m⁻² s⁻¹. To induce low-temperature stress, the seedlings were incubated at 4°C in water for up to 6 h. High-salinity stress was induced by incubation in 400 mM NaCl for 2 h at 28°C. To simulate drought stress, the plants were air dried for 2 h at 28°C. F₀/F₅ and F_m values were then measured as described previously (Oh et al., 2008).

**Rice 3′-Tiling Microarray Analysis**

Expression profiling was conducted using the Rice 3′-Tiling microarray as described previously (Oh et al., 2009). Transgenic and NT rice (cv Nipponbare) seeds were germinated in soil and grown in a greenhouse (16-h-light/8-h-dark cycle) at 22°C. To identify stress-inducible NAC genes in rice, total RNA (100 μg) was prepared from 14-d-old leaves of plants subjected to drought, high-salinity, ABA, and low-temperature stress conditions. For high-salinity and ABA treatments, the 14-d-old seedlings were transferred to a nutrient solution containing 400 mM NaCl or 100 μM ABA for the indicated periods.
containing 400 mM NaCl or 100 μM ABA for 2 h in the greenhouse under continuous light of approximately 1,000 μmol m⁻² s⁻¹. For drought treatment, 14-d-old seedlings were air dried for 2 h also under continuous light of approximately 1,000 μmol m⁻² s⁻¹. For low-temperature treatment, 14-d-old seedlings were exposed at 4°C in a cold chamber for 6 h also under continuous light of 150 μmol m⁻² s⁻¹. For identification of genes up-regulated in RCc3: OsNAC10 and GOS2:OsNAC10 plants, total RNA (100 μg) was prepared from root and leaf tissues of 14-d-old transgenic and NT rice seedlings (cv Nipponbare) grown under normal growth conditions.

Quantitative PCR Analysis

Total RNA was prepared as reported previously (Kim et al., 2009). For quantitative real-time PCR experiments, a SuperScript III Platinum One-Step Quantitative RT-PCR system (Invitrogen) was used. For PCR, a master mix of reaction components was prepared as reported previously (Oh et al., 2009) using Platinum SYBR Green qPCR SuperMix-UDG (Invitrogen). Thermocycling and fluorescence detection were performed using a Stratagene MX3000p Real-Time PCR machine (Stratagene). PCR was performed at 95°C for 10 min, followed by 40 cycles of 94°C for 30 s, 57°C for 30 s, and 68°C for 1 min. To validate our quantitative PCR results, we repeated each experiment three times. The primer pairs are listed in Supplemental Table S5.

Drought Treatments and Grain Yield Analysis of Rice Plants in the Field for Two Years (2008 and 2009)

To evaluate yield components of transgenic plants under normal field conditions, three independent T4 (2008) and T5 (2009) homozygous lines of the RCc3:OsNAC10 and GOS2:OsNAC10 plants, together with NT controls, were transplanted to a paddy field at the Rural Development Administration, Suwon, Korea. A randomized design was employed with two (2008) and three (2009) replicates. At 25 d after sowing, the seedlings were randomly transplanted with 15–30 cm spacing and a single seedling per hill. Fertilizer was applied at 70:40:70 (nitrogen:phosphorus:potassium) kg ha⁻¹ after the last paddling and 45 d after transplantation. Yield parameters were scored for 20 (2008) and 30 (2009) plants per transgenic line. Plants located at borders were excluded from data scoring.

To evaluate yield components of transgenic plants under drought field conditions, three independent T4 (2008) and T5 (2009) homozygous lines of each of the RCc3:OsNAC10 and GOS2:OsNAC10 plants and NT controls were transplanted to a removable rain-off shelter (located at Myongji University, Yongin, Korea) with a 1-m-deep container filled with natural paddy soil.

The experimental design, transplanting spacing, use of fertilizer, drought treatments, and scoring of agronomic traits were as described (Oh et al., 2009). When the plants grown under normal and drought conditions had reached maturity and the grains had ripened, they were harvested and threshed by hand (separation of seeds from the vegetative parts of the plant). The unfilled and filled grains were then taken apart, independently counted using a Countmate MC1000H (Prince Ltd.), and weighed. The following agronomic traits were scored: flowering date, panicle length, number of fillers, number of panicles, spikelets per panicle, filling rate (%), total grain weight (g), and 1,000 grain weight (g). The results from three independent lines were separately analyzed by one-way ANOVA and compared with those of the NT controls.

The ANOVA was used to test the null hypothesis of equal means of transgenic lines and NT controls (P < 0.05). SPSS version 16.0 was used to perform these statistical analyses.

Microscopic Examination of Roots

Roots of transgenic and NT plants at the panicle heading stage were fixed with modified Karnovsky’s fixative, consisting of 2% (v/v) glutaraldehyde and 2% (v/v) paraformaldehyde in 0.1 mol M NaCl or 100 μM ABA for 2 h in the greenhouse under continuous light of approximately 1,000 μmol m⁻² s⁻¹. For dehydration, 14-d-old seedlings were air dried for 2 h also under continuous light of approximately 1,000 μmol m⁻² s⁻¹. For low-temperature treatment, 14-d-old seedlings were exposed at 4°C in a cold chamber for 6 h also under continuous light of 150 μmol m⁻² s⁻¹. For identification of genes up-regulated in RCc3: OsNAC10 and GOS2:OsNAC10 plants, total RNA (100 μg) was prepared from root and leaf tissues of 14-d-old transgenic and NT rice seedlings (cv Nipponbare) grown under normal growth conditions.

knife by an ultramicrotome (MT-X; RMC). The sections were stained with 1% toluidine blue and observed and photographed with a light microscope.

Supplemental Data

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

Supplemental Figure S1. Phylogenetic relationship of the rice NAC family.

Supplemental Figure S2. Regulated genes in roots and leaves of RCc3: OsNAC10 and GOS2:OsNAC10 plants under normal and stress conditions.

Supplemental Table S1. Up-regulated root-expressed genes in either RCc3:OsNAC10 or GOS2:OsNAC10 plants in comparison with NT controls.

Supplemental Table S2. Up-regulated leaf-expressed genes in GOS2: OsNAC10 plants in comparison with NT controls.

Supplemental Table S3. Agronomic traits of the RCc3:OsNAC10 and GOS2:OsNAC10 transgenic rice plants under normal field conditions.

Supplemental Table S4. Agronomic traits of the RCc3:OsNAC10 and GOS2:OsNAC10 transgenic rice plants under field drought conditions.

Supplemental Table S5. Primer list for PCR.

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LITERATURE CITED


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