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OsNAC5 overexpression enlarges root diameter in rice plants leading to enhanced drought tolerance and increased grain yield in the field

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Summary

Drought conditions are among the most serious challenges to crop production worldwide. Here, we report the results of field evaluations of transgenic rice plants overexpressing *OsNAC5*, under the control of either the root-specific (*RCc3*) or constitutive (*GOS2*) promoters. Field evaluations over three growing seasons revealed that the grain yield of the *RCc3:OsNAC5* and *GOS2:OsNAC5* plants were increased by 9%–23% and 9%–26% under normal conditions, respectively. Under drought conditions, however, *RCc3:OsNAC5* plants showed a significantly higher grain yield of 22%–63%, whilst the *GOS2:OsNAC5* plants showed a reduced or similar yield to the nontransgenic (NT) controls. Both the *RCc3:OsNAC5* and *GOS2:OsNAC5* plants were found to have larger roots due to an enlarged stele and aerenchyma at flowering stage. Cell numbers per cortex layer and stele of developing roots were higher in both transgenic plants than NT controls, contributing to the increase in root diameter. The root diameter was enlarged to a greater extent in the *RCc3:OsNAC5*, suggesting the importance of this phenotype for enhanced drought tolerance. Microarray experiments identified 25 up-regulated genes by more than three-fold ($P < 0.01$) in the roots of both transgenic lines. Also identified were 19 and 18 up-regulated genes that are specific to the *RCc3:OsNAC5* and *GOS2:OsNAC5* roots, respectively. Of the genes specifically up-regulated in the *RCc3:OsNAC5* roots, *GLP*, *PDX*, *MERI5* and *O-methyltransferase* were implicated in root growth and development. Our present findings demonstrate that the root-specific overexpression of *OsNAC5* enlarges roots significantly and thereby enhances drought tolerance and grain yield under field conditions.

Introduction

Upon exposure of plants to drought, high salinity and low temperature, many genes are induced as an adaptive response to such adverse conditions (Bray, 2004; Fowler and Thomashow, 2002; Maggio *et al.*, 2006; Rabbani *et al.*, 2003; Seki *et al.*, 2002; Yamaguchi-Shinozaki and Shinozaki, 2006). One such group of genes includes the transcription factors (TFs) that regulate key downstream genes. The rice and Arabidopsis genomes encode over 1300 TFs, 45% of which are reported to be from gene families specific to plants (Kikuchi *et al.*, 2003; Riechmann *et al.*, 2000). NAC (*NAM*, *ATAF* and *CUC*) domain-containing proteins constitute one large plant-specific family, with 151 and 117 predicted members (Nuruzzaman *et al.*, 2010) in rice and Arabidopsis, respectively. NAC domains located at the N-terminus comprise approximately 160 amino acid residues (Ooka *et al.*, 2003), whereas the C-terminal regions are highly divergent, conferring diverse transcriptional activities (Xie *et al.*, 2000; Yamaguchi *et al.*, 2008). The earliest reported *NAC* genes include *NAM* from petunia (*Petunia hybrida*), which determines the position of the shoot apical meristem (SAM) (Souer *et al.*, 1996), and *CUC2* from Arabidopsis, which 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 signalling to promote lateral root development (Xie *et al.*, 2000).

Many other *NAC* genes have been implicated in diverse cellular processes in various plant species, such as hormone signal pathways (Greve *et al.*, 2003) and development (Peng *et al.*, 2009). NAC proteins may also function in homodimers and/or heterodimers in plants. Arabidopsis NAC1 and ANAC form homodimers (Ernst *et al.*, 2004; Xie *et al.*, 2000), Brassica BnNAC14 forms heterodimers with BnNAC3, BnNAC5-8, BnNAC5-11 and BnNAC485 (Hegedus *et al.*, 2003), and OsNAC5 forms homodimers and heterodimers with other OsNACs (Jeong *et al.*, 2009; Takasaki *et al.*, 2010). Genes in the ATAF subfamily (Ooka *et al.*, 2003), such as ATAF1 and 2 (Aida *et al.*, 1997) from Arabidopsis, are induced by pathogen attack and wounding. Recently, *AtNAC072* (*RD29*), *AtNAC019*, *AtNAC055* (Fujita *et al.*, 2004; Tran *et al.*, 2004), and *ANAC102* (Christianson *et al.*, 2009) from Arabidopsis, *BnNAC* from *Brassica napus* (Hegedus *et al.*, 2003), and *SNAC1* (Hu *et al.*, 2006), *SNAC2/OsNAC6* (Hu *et al.*, 2008; Nakashima *et al.*, 2007), *OsNAC5* (Song *et al.*, 2011; Sperotto *et al.*, 2009; Takasaki *et al.*, 2010; Zheng *et al.*, 2009) and *OsNAC10* (Jeong *et al.*, 2010) from rice were shown to be involved in responses to various environmental stresses. Interestingly, seven *NAC* members including *CUC1* and *CUC2*

have also been shown to be regulated post-transcriptionally by interacting with miR164 (Gustafson *et al.*, 2005; Raman *et al.*, 2008). Together with miR164, NAC domain TFs regulate diverse processes during plant development that includes pattern formation in the embryo and flower (Larue *et al.*, 2009), boundaries for the separation of organs (Vroemen *et al.*, 2003; Weir *et al.*, 2004), lateral roots (D'haeseleer *et al.*, 2011), SAM (Vroemen *et al.*, 2003), responses to biotic and abiotic stress (Hegedus *et al.*, 2003; Tran *et al.*, 2004), senescence (Kim *et al.*, 2009) and the transportation of mRNA via phloem (Kehr and Buhtz, 2007).

Drought is one of the major constraints to rice yields worldwide. In particular, exposure to drought conditions during the panicle development stage results in a delayed flowering time, a reduced number of spikelets and poor grain filling (Ekanayake *et al.*, 1989; O'Toole and Namuco, 1983). To date, a number of studies have suggested that the overexpression of stress-related genes improves drought tolerance in rice under greenhouse conditions (Garg *et al.*, 2002; Hu *et al.*, 2006, 2008; Ito *et al.*, 2006; Jang *et al.*, 2003; Nakashima *et al.*, 2007; Oh *et al.*, 2007; Xu *et al.*, 1996). However, very few reports have shown an improvement in grain yield under field conditions (Hu *et al.*, 2006; Jeong *et al.*, 2010; Oh *et al.*, 2009; Wang *et al.*, 2005).

Recently, the stress-responsive gene *OsNAC5* was reported (Song *et al.*, 2011; Takasaki *et al.*, 2010). *OsNAC5*-overexpressing transgenic plants had increased tolerance to drought, high salinity and low temperature. The studies, however, were limited to tolerance only at the vegetative stage and the physiological mechanisms under field drought conditions at the reproductive stage remained elusive.

In our current study, a genome-wide analysis of rice *NAC* TFs s was conducted to identify genes that improve grain yield and tolerance to environmental stress. A total of 18 stress-inducible *OsNAC*s (Jeong *et al.*, 2010) were prescreened for enhanced drought tolerance when overexpressed in rice. We here report the results of field evaluations of transgenic rice plants overexpressing *OsNAC5*, one of the effective members of this family that was selected in a prescreening. The overexpression of *OsNAC5* under the control of the root-specific (*RCc3*) and constitutive (*GOS2*) promoters improved rice plant tolerance to drought and high salinity during the vegetative stage of growth. More importantly, the root-specific overexpression of this gene significantly enhanced drought tolerance at the reproductive stage of growth via enlarged roots, with a concomitant increase of grain yield.

Results

The transgenic overexpression of *OsNAC5* increases rice plant tolerance to drought and high-salinity

To investigate the transcript levels of *OsNAC5* under stress conditions, we performed RNA gel-blot analysis of the leaf and root tissues of 14-day-old rice seedlings exposed to high salinity, drought, ABA and low temperature (Figure 1a). *OsNAC5* expression in both the leaf and root tissues was significantly induced by drought, high-salinity and ABA, but not by low-temperature conditions. The *OsNAC5* mRNA levels started to increase at 0.5 h after drought and salt treatments and peaked at 2 h post-treatment, whilst these transcript levels gradually increased for up to 6 h after exposure to exogenous ABA. Levels of *OsNAC5* transcript were not increased upon exposure to cold-stress up to 6 h, but could be responsive at prolonged exposure. Earlier studies (Song *et al.*, 2011; Takasaki *et al.*, 2010) have shown that the *OsNAC5* transcripts started to accumulate after exposure to low temperature for 24 h. It is possible that transcripts of *OsNAC5* were accumulated only in small quantity at early time after exposure to low temperature, which was under detection limit in our RNA blot analysis. For the overexpression of *OsNAC5* in rice plants, two expression vectors, *RCc3:OsNAC5* and *GOS2:OsNAC5*, were generated by separately fusing the cDNA of *OsNAC5* with that of *RCc3* (Xu *et al.*, 1995) and *GOS2* (de Pater *et al.*, 1992) to enable root-specific and whole-body expression, respectively. These expression vectors were then transformed into rice (*Oryza sativa* cv Nipponbare) using the *Agrobacterium*-mediated method (Hiei *et al.*, 1994), and 15–20 transgenic plants were produced per construct. T_{1-7} seeds from these transgenic lines that grew normally with no stunting were collected, and three independent T_{5-7} homozygous lines of both *RCc3:OsNAC5* and *GOS2:OsNAC5* plants were selected for further analysis. To determine the expression levels of *OsNAC5* in the transgenic plants, RNA gel-blot analysis of the leaf and root tissues of 14-day-old seedlings grown under normal growth conditions was performed. Increased levels of *OsNAC5* expression were detected in the roots only of the *RCc3:OsNAC5* plants and in both the leaves and roots of the *GOS2:OsNAC5* plants, but not in NT and nullizygous (segregants with no transgene inserts) plants (Figure 1b).

To evaluate the tolerance of these transgenic rice plants to drought stress, 1-month-old transgenic and NT control plants grown in a greenhouse were subjected to drought stress by withholding water. Over the time course of these drought treatments, both sets of transgenic plants performed better than the NT controls and showed delayed symptoms of stress-induced damage, such as wilting and leaf-rolling with the concomitant loss of chlorophyll (Figure 2a). The transgenic plants also recovered better during re-watering for up to 7 days. Significantly, the survival rates of the transgenic plants ranged from 60% to 80%, whereas the NT control plants showed no signs of recovery. To further verify this enhanced stress tolerance, we measured the Fv/Fm values that are an indicator of the photochemical efficiency of photosystem II (PSII) in a dark-adapted state. The leaf discs of 2-week-old transgenic and NT control plants were subjected to drought, high-salinity and low-temperature conditions for the indicated times. The average F_v/F_m value of nonstressed plants was approximately 0.8. At the initial stages of the drought (0.5 h) and high-salinity (2 h) conditions, the F_v/F_m levels of the *RCc3:OsNAC5* and *GOS2:OsNAC5* plants were higher than the NT controls by 15%–22% ($P < 0.01$; Figure 2b). Under extended drought (2 h) and high-salinity (6 h) stress as well as low-temperature conditions, however, these levels remained similar to those of the NT controls, suggesting a moderate level of tolerance in the transgenic plants.

The JIP test, named after the polyphasic fluorescence rise of the O-J-I-P transients following illumination of dark-adapted plants with actinic light, can provide information on the changes in the energetic connectivity in the antennas of the PSII units when plants are exposed to environmental stress (Redillas *et al.*, 2011a, b). This connectivity can be illustrated through normalization between F_O (50 μs) and F_K (300 μs). By calculating the difference kinetics between transgenic and NT plants, an L-band around 150 μs is produced. This band is negative (or positive) when the connectivity of the plants is higher (or lower) than that of the untreated NT controls. This parameter is undetectable using the F_v/F_m analysis, which also measures the chlorophyll *a*

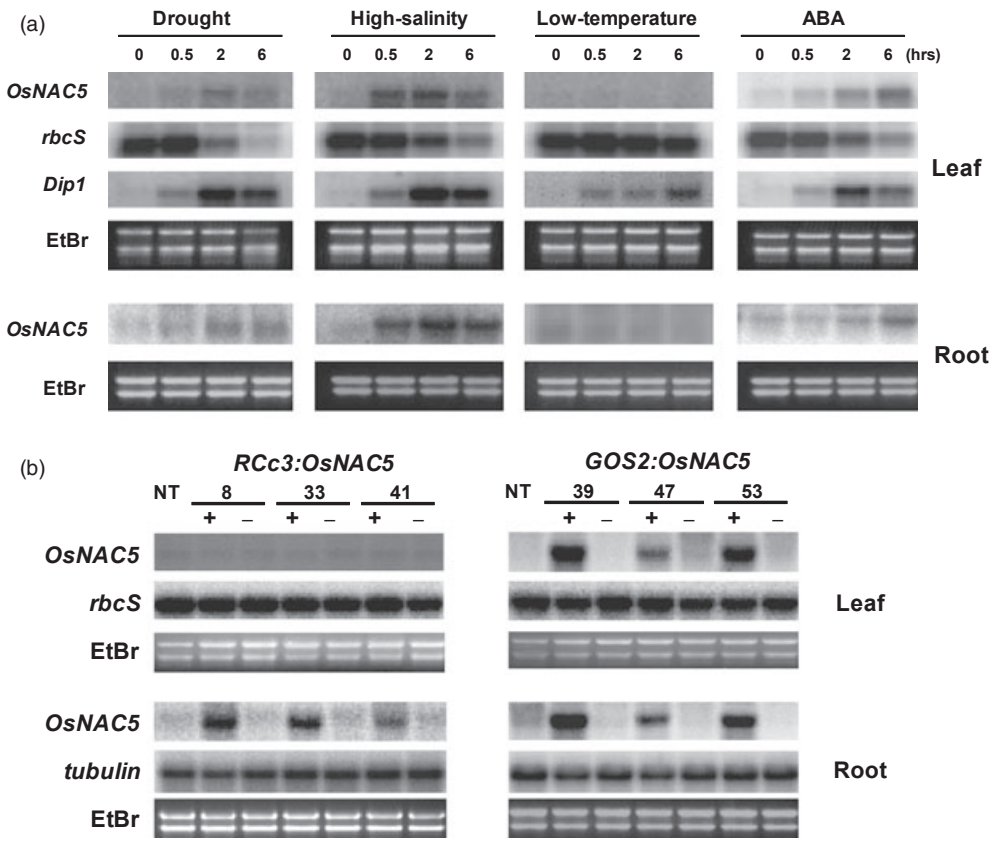

Figure 1 RNA gel-blot analysis of *OsNAC5*. (a) Ten micrograms of total RNA was prepared from the leaf and root tissues of 14-day-old seedlings exposed to drought, high salinity, ABA or low temperature for the indicated time periods. For drought stress, the seedlings were air-dried at 28 °C; for high-salinity stress, the seedlings were exposed to 400 mM NaCl at 28 °C; for low-temperature stress, the seedlings were exposed to 4 °C; for ABA treatment, the seedlings were exposed to a solution containing 100 μM ABA. Total RNAs were blotted and hybridized with *Dip1* (Oh *et al.*, 2005) and *rbcS* (Jang *et al.*, 1999) probes, which were used as markers for up- and down-regulation, respectively, following stress treatments (Jeong *et al.*, 2010) and then reprobed with *OsNAC5* gene-specific region. Ethidium bromide (EtBr) staining was used to determine equal loading of the *RNA*s. (b) *RNA* gel-blot analyses using total *RNA* preparations from the roots and leaves of three homozygous T_5 lines of *RCc3:OsNAC5* and *GOS2:OsNAC5* plants, respectively, and of nontransgenic (NT) control plants. The blots were hybridized with *OsNAC5* gene-specific probes and also reprobed for *rbcS* and *tubulin*. Ethidium bromide staining was used to determine equal loading of the RNAs. (–) means nullizygous (segregants without transgene) lines, (+) means transgenic lines.

fluorescence of plants. We performed the JIP test on the transgenic and NT control plants at the reproductive stage and found that both of the transgenic lines had higher connectivity than the NT controls under drought conditions (Figure 2c). More specifically, the connectivity was found to be highest in the *RCc3:OsNAC5* plants followed by the *GOS2:OsNAC5* lines, thus revealing differences in drought tolerance at the reproductive stage. This drought tolerance can also be seen through a decline in the end electron acceptors for drought-treated plants when compared with plants grown under normal conditions (Figure 2d).

The overexpression of *OsNAC5* increases grain yield under both normal and drought conditions

The field performance of *RCc3:OsNAC5* and *GOS2:OsNAC5* plants was evaluated for three growing seasons in a paddy field under both normal and drought conditions. Three independent T_5 (2009), T_6 (2010) and T_7 (2011) homozygous lines of *RCc3:OsNAC5* and *GOS2:OsNAC5* plants, together with NT controls, were transplanted to a paddy field and grown to maturity. Yield parameters were scored for 30 plants per transgenic line from three replicates. The data sets obtained from 3 years of field testing were generally similar with some variations and the total grain weights of the *RCc3:OsNAC5*, and the *GOS2:OsNAC5* plants were increased by 9%–23% and 9%–26% ($P < 0.05$), respectively. This increased yield for both transgenic plants was coupled with an increased number of spikelet per panicle and increased total number of spikelets with a filling rate similar to that of the NT controls (Table 1, Table S1). We did not include nullizygous plants for agronomic trait controls due to no significant statistical differences ($P < 0.05$) in yield parameters between NT and nullizygous plants (Table S3).

To further test the *RCc3:OsNAC5* and *GOS2:OsNAC5* plants under drought conditions, three independent T_5 (2009), T_6 (2010) and T_7 (2011) lines of each transgenic plant were transplanted to a refined field equipped with a movable rain-off shelter. The plants were exposed to drought stress at the panicle heading stage (from 10 days before heading to 10 days after heading). Following exposure to drought stress until complete leaf-rolling had occurred, plants were irrigated overnight and immediately subjected again to a second round of drought conditions until complete leaf-rolling again occurred. Upon completion of these drought treatments, plants were irrigated to allow recovery until the seed maturation stages. The level of drought stress imposed

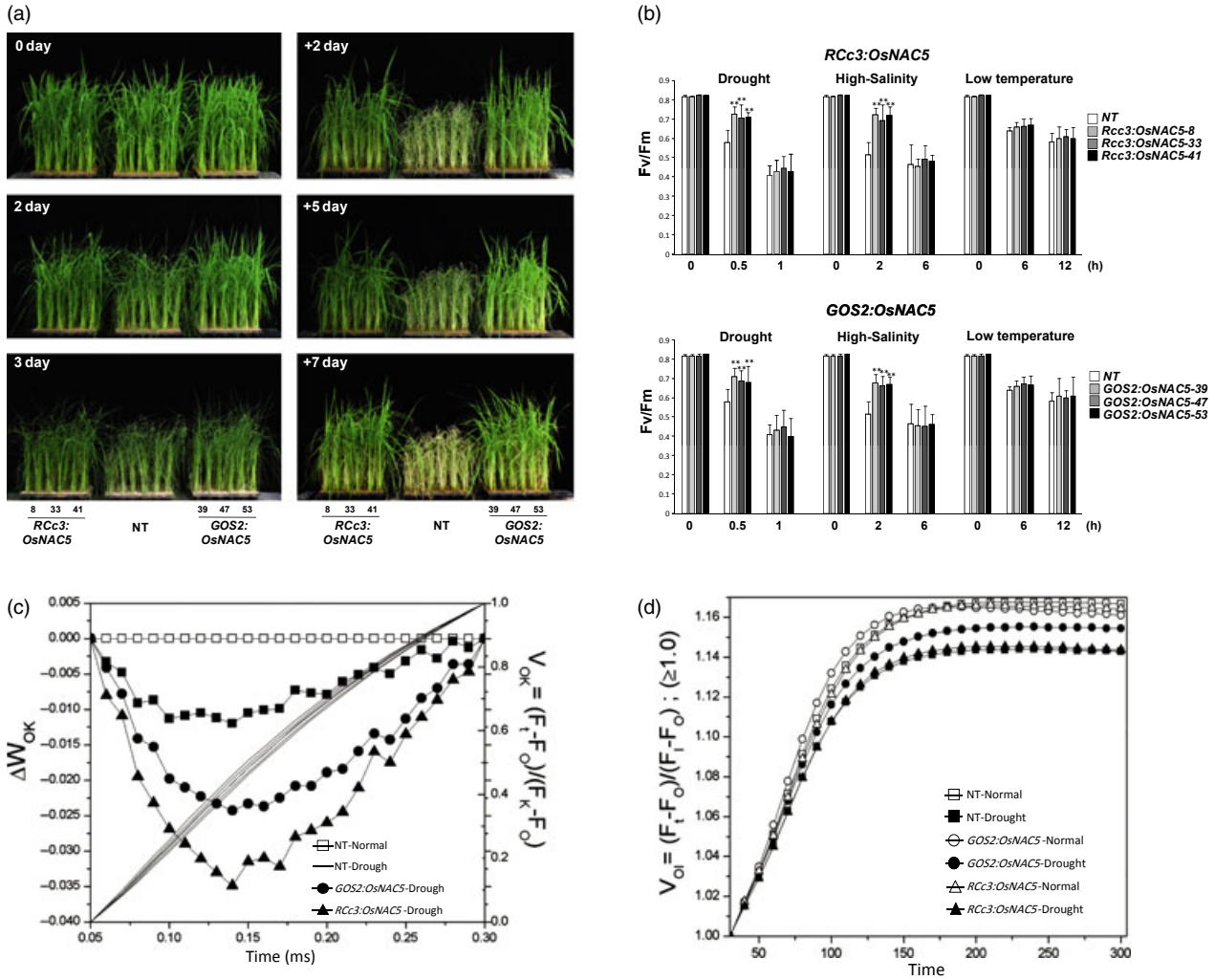

Figure 2 Stress tolerance of *RCc3:OsNAC5* and *GOS2:OsNAC5* plants. (a) The appearance of transgenic plants during drought stress. Three independent homozygous T_6 lines of *RCc3:OsNAC5* and *GOS2:OsNAC5* plants and nontransgenic (NT) controls were grown for 4 weeks, subjected to 3 days of drought stress and then 7 days of re-watering in the greenhouse. Images were taken at the indicated time points. '+' denotes the number of re-watering days under normal growth conditions. (b) Changes in the chlorophyll fluorescence (F_v/F_m) of rice plants under drought, high salinity and low-temperature stress conditions. Three independent homozygous T_6 lines of *RCc3:OsNAC5* and *GOS2:OsNAC5* plants and NT controls grown in MS medium for 14 days were subjected to various stress conditions as described in the Materials and Methods section. After each stress treatment, the F_v/F_m values were measured using a pulse modulation fluorometer (mini-PAM, Walz, Germany). All plants were grown under continuous light of 150 μmol/m^2/s prior to stress induction. Each data point represents the mean ± SE of triplicate experiments (n = 10). Asterisk (**) indicates a significant difference ($P < 0.01$). (c) L-bands of the plants grown under drought conditions revealed by kinetic differences at the F_O to F_K in accordance with the equation $\Delta W_{OK} = V_{OKsample} - V_{OKcontrol}$; left axis. Double normalization at the O to K phase was calculated by $V_{OK} = (F_t - F_O)/(F_K - F_O)$; right axis. (d) Events for $V_{OI} \geq 1.0$ illustrating the differences in the pool size of the end electron acceptors calculated as $V_{OI} = (F_t - F_O)/(F_t - F_O)$ under both normal and drought conditions.

under the rain-off shelter was equivalent to that which causes a 60% reduction in the total grain weight obtained under normal growth conditions, as evidenced by the NT plant yields under normal and drought conditions (Tables S1 and S2). Statistical analysis of the yield parameters scored for three growing seasons showed that the decrease in grain yield under drought conditions was significantly smaller in the *RCc3:OsNAC5* plants than that observed in either *GOS2:OsNAC5* or NT controls. Specifically, in the drought-treated *RCc3:OsNAC5* plants, the numbers of spikelets and/or the filling rates were higher than in the drought-treated NT plants, which resulted in an increase in the total grain weight by 33%–63% (2009), 22%–48% (2010) and 33%–57% (2011), depending on transgenic line (Table 1, Table S2). In the drought-treated *GOS2:OsNAC5* plants, in contrast, the total grain weight was less than (2009) or remained similar to (2010 and 2011) the drought-treated NT controls. Given the similar levels of drought tolerance during the vegetative stage in the *RCc3:OsNAC5* and *GOS2:OsNAC5* plants, the differences in total grain weight under field drought conditions were rather unexpected. These observations prompted us to examine the root architecture of transgenic plants. We measured the root volume, length, dry weight and diameter of *RCc3:OsNAC5*, *GOS2:OsNAC5* and NT plants grown to the heading stage of reproduction. As shown in Figure 3a,b, the root diameter of the *RCc3:OsNAC5* plants was larger than that of the NT control plants by 30% ($P < 0.01$). Microscopic analysis of cross-sectioned *RCc3:OsNAC5* roots further revealed that this increase in root diameter was due to the enlarged stele and aerenchyma. In

Table 1 Agronomic traits of *RCc3:OsNAC5* and *GOS2:OsNAC5* plants grown in the field under both normal and drought conditions

	Filling rate (%)						Total grain weight (g)					
	2009 (T5)		2010 (T6)		2011 (T7)		2009 (T5)		2010 (T6)		2011 (T7)	
Constructs	Normal	Drought	Normal	Drought	Normal	Drought	Normal	Drought	Normal	Drought	Normal	Drought
NT (Nipponbare)	91.29	47.03	82.74	47.62	86.76	49.72	21.41	8.55	27.82	10.09	18.97	8.45
RCc3:OsNAC5-8(+)	90.22	59.43*	82.77	52.25 *	91.52 *	60.40 *	24.52 *	12.22 *	32.00 *	12.40 *	22.35 *	13.14
%Δ	−1.17	26.36	0.04	9.73	5.49	21.48	14.52	42.81	15.01	22.91	17.84	55.43
RCc3:OsNAC5-33(+)	93.42 *	68.63 *	84.26 *	63.05 *	91.51 *	63.77 *	23.99 *	13.97 *	31.40 *	14.97 *	22.05 *	13.29
%Δ	2.33	45.91	1.83	32.40	5.47	28.27	12.01	63.30	12.85	48.35	16.24	57.22
RCc3:OsNAC5-41(+)	92.83 *	54.95 *	85.20 *	46.47 *	89.41 *	56.19 *	23.41 *	11.39 *	31.00 *	12.38 *	23.01 *	11.25
%Δ	1.69	16.83	2.98	−2.41	3.05	13.01	9.31	33.16	11.42	22.69	21.30	33.08
GOS2:OsNAC5-39(+)	92.05	37.65	83.11	47.88	87.23	53.95	24.47 *	7.70	30.51 *	10.64	23.49 *	10.88
%Δ	0.84	−19.95	0.45	0.95	0.53	8.50	14.26	−10.01	9.66	5.45	23.85	28.68
GOS2:OsNAC5-47(+)	90.84	37.81 *	85.28	49.59	90.59 *	53.40	24.38 *	6.52 *	35.20 *	11.31	23.07 *	10.59
%Δ	−0.50	−19.61	3.08	4.15	4.41	7.41	13.84	−23.75	26.51	12.11	21.62	25.25
GOS2:OsNAC5-53(+)	81.40 *	22.18 *	72.81 *	41.31	89.22 *	51.88	21.91	3.72 *	28.30	10.28	22.00 *	9.17
%Δ	−10.84	−52.84	−12.00	−13.25	2.83	4.34	2.32	−56.54	1.73	1.93	15.99	8.47

Filling rate and total grain weight of three independent homozygous T_5, T_6 and T_7 lines of *RCc3:OsNAC5*and *GOS2:OsNAC5* plants and corresponding nontransgenic (NT) controls, grown under both normal and drought conditions. Each parameter value represents the mean (n = 30) for *RCc3:OsNAC5* and *GOS2:OsNAC5* plants and respective NT controls. Percentage differences (%Δ) between the values for the *RCc3:OsNAC5* and *GOS2:OsNAC5* plants and NT controls are listed.
*Significant difference ($P < 0.05$).

particular, the metaxylem (Me), a major portion of the stele, and the aerenchyma (Ae), a tissue that results from cortical cell death, were larger in the *RCc3:OsNAC5* plants compared with the NT roots (Figure 3c). The sizes of the metaxylem and aerenchyma have been previously found to correlate with drought tolerance at the reproductive stage (Yambao *et al.*, 1992; Zue *et al.*, 2010). In 2-month-old *RCc3:OsNAC5* roots, increase in cell numbers per cortex layer was evident as compared with those of the NT roots (Figure 3d,e). The increase in cell numbers is also true for stele of *RCc3:OsNAC5* roots (Figure 3d). Thus, our results suggest that root-specific overexpression of *OsNAC5* increase the root diameter that contributed to the increase in grain yield of the *RCc3:OsNAC5* plants under normal and/or drought conditions.

Identification of genes up-regulated following *OsNAC5* overexpression

To screen for genes that are up-regulated by the overexpression of *OsNAC5*, we performed expression profiling of the *RCc3:OsNAC5* and *GOS2:OsNAC5* plants in comparison with NT controls. This profiling was conducted using the rice 3′-tiling microarray with RNA samples extracted from roots of 14-day-old plants of each type grown under normal conditions. Each data set was obtained from two biological replicates. Statistical analysis using one-way ANOVA identified 25 target genes that were up-regulated following *OsNAC5* overexpression by more than three-fold in both transgenic roots compared with the NT controls ($P < 0.01$). In addition, we identified 19 and 18 target genes in the same analysis, respectively, that were up-regulated specifically in the *RCc3:OsNAC5* and *GOS2:OsNAC5* roots (Table 2). Microarray experiments that were previously performed (GEO accession number GSE31874) had revealed a total of 23 of 62 target genes (8, 8 and 7 genes that were common, *RCc3:OsNAC5*–specific and *GOS2:OsNAC5*–specific, respectively) to be stress-inducible under drought, high-salinity, cold and ABA stress conditions (Table 2). The up-regulated target genes common to both transgenic roots include *9-cis-epoxycarotenoid dioxygenase* (*NCED*, Tan *et al.*, 1997), *Calcium-transporting ATPase* (Knight, 2000), and *Cinnamoyl CoA reductase* (Fan *et al.*, 2006; Goujon *et al.*, 2003; Jones *et al.*, 2001; Tamasloukht *et al.*, 2011). In addition, the Germin-like protein (*GLP*, Yin *et al.*, 2009), Pyridoxin biosynthesis protein (*PDX*, Titiz *et al.*, 2006), Meristem protein (*MERI5*, Verica and Medford, 1997) and *O-methyltransferases* (Held *et al.*, 1993; Yamaguchi and Sharp, 2010) genes involved in cell growth and development were found in our analysis to be up-regulated specifically in *RCc3:OsNAC5* roots, suggesting a role in altering the root architecture. We selected nine target genes and verified their *OsNAC5*-dependent expression patterns in *RCc3:OsNAC5* and *GOS2:OsNAC5* roots under normal growth conditions by qPCR (Figure 4). Previously, we have reported that the root-specific expression of the *OsNAC10* enhanced drought tolerance via similar increase in root diameter (Jeong *et al.*, 2010). We, therefore, compared expression patterns of *OsNAC5* target genes with those of the *OsNAC10* target genes, finding only 17 genes (of 62 *OsNAC5* target genes) to be common to both *OsNAC5* and *OsNAC10* roots (Figure 4, Table S4). In addition, expression specificities of those 17 genes were different between the *OsNAC5* and *OsNAC10* roots. For example, *Cinnamoyl CoA reductase* was up-regulated in both *RCc3:OsNAC5* and *GOS2:OsNAC5* roots, whereas it was up-regulated only in *RCc3:OsNAC10* roots (Figure 4). Thus, *OsNAC5* up-regulates its target genes independently of *OsNAC10*, hence increases root diameter in a different mechanism.

Discussion

In this study, we found that *OsNAC5* is up-regulated in response to drought, high salinity and ABA stress. Moreover, the overexpression of the gene under the control of the constitutive (*GOS2*) and root-specific (*RCc3*) promoters in transgenic rice was found to enhance plant tolerance to drought and high salinity at the

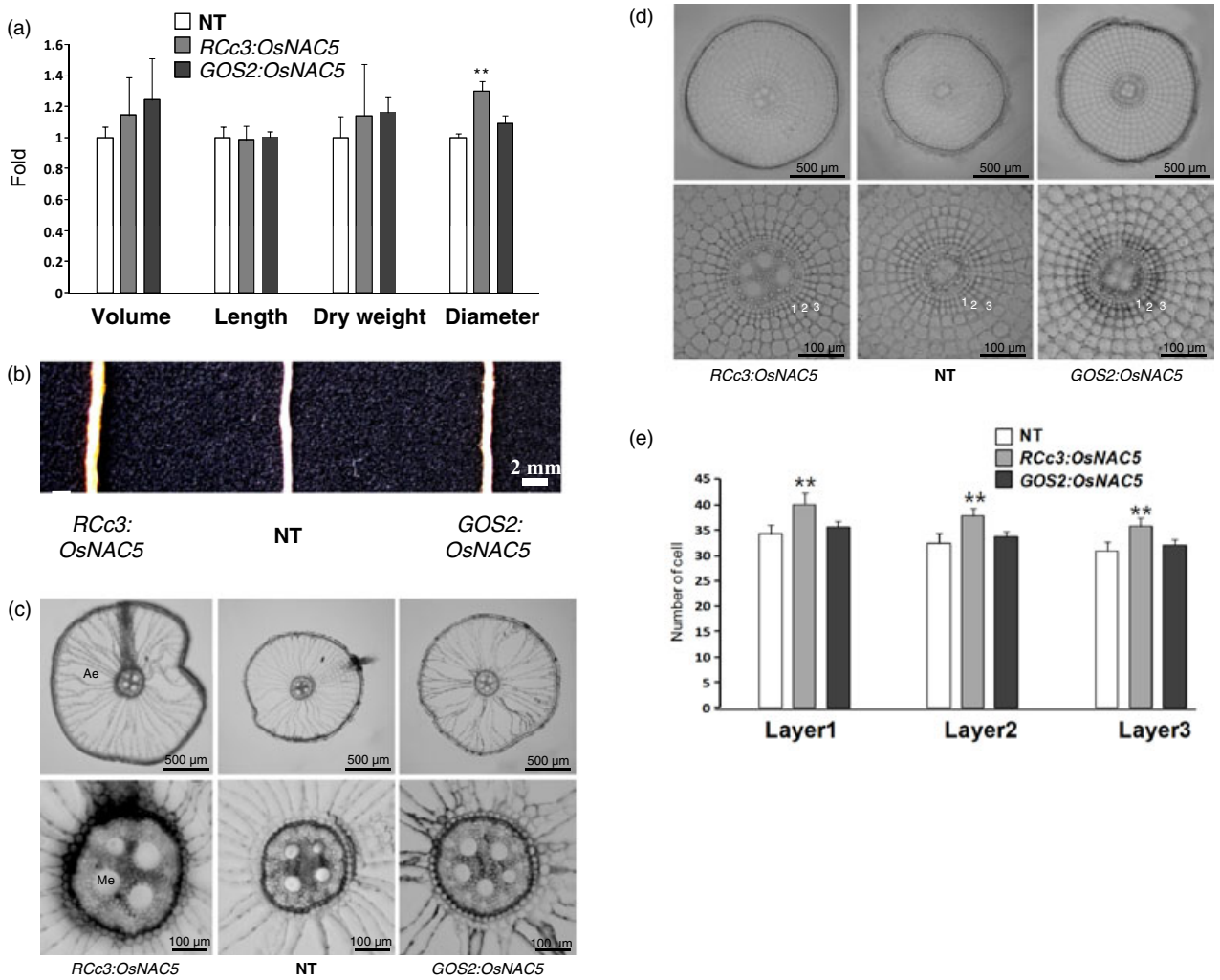

Figure 3 Differences in the root growth of *RCc3:OsNAC5* and *GOS2:OsNAC5* plants. (a) The root volume, length, dry weight and diameter in *RCc3:OsNAC5* and *GOS2:OsNAC5* plants were normalized to those of nontransgenic (NT) control roots. Asterisk (**) indicates a significant difference ($P < 0.01$). Values for the volume, length and dry weight are the means ± SD of five plants whilst 50 roots (10 roots from each of five plants) were used for the diameter. (b) A representative root of the *RCc3:OsNAC5*, *GOS2:OsNAC5* and NT control plants that were grown to the heading stage of reproduction. Scale bars, 2 mm. (c) Light microscopic images of cross-sectioned *RCc3:OsNAC5*, *GOS2:OsNAC5* and NT roots (10 cm down from the ground surface level) during the panicle heading stage. The position of the metaxylem vessel (Me) and aerenchyma (Ae) are indicated. Scale bars, 500 μm in the upper panels and 100 μm in the lower panels. (d) Light microscopic images of cross-sectioned of 2-month-old *RCc3:OsNAC5*, *GOS2:OsNAC5* and NT roots (1 cm above the root tip). The numbers (1, 2 and 3) indicate cortex layers for counting cell numbers. Scale bars, 500 μm in the upper panels and 100 μm in the middle and lower panels. (e) Number of cells per cortex layer of 2-month-old *RCc3:OsNAC5*, *GOS2:OsNAC5* and NT roots ($n = 10$). Asterisks (**) indicate a significant difference ($P < 0.01$).

vegetative stage of growth. Consistent with our current findings for the *RCc3:OsNAC5* and *GOS2:OsNAC5* plants, the overexpression of *OsNAC5* using the maize ubiquitin (Takasaki *et al.*, 2010) and the CaMV 35S promoter (Song *et al.*, 2011) in rice has been shown previously to confer tolerance to drought and high-salinity stress. In our present analysis, we used three independent homozygous T_5, T_6 and T_7 lines of the *RCc3:OsNAC5* and *GOS2:OsNAC5* plants to evaluate agronomic traits for three growing seasons (2009, 2010 and 2011) in three different paddy fields. Both transgenic plant types showed a significantly increased grain yield under normal growth conditions. Under field drought conditions, in contrast, the *RCc3:OsNAC5* plants performed much better than the *GOS2:OsNAC5* plants, showing grain yield enhancements of 33%–63% (2009), 22%–48% (2010) and 33%–57% (2011) over the NT controls. In our current analyses, before field evaluations attempted, we prescreened the *RCc3:OsNAC5* and *GOS2:OsNAC5* plants from T_1 through T_4 at normal field conditions, excluding plants with morphological alterations such as abnormal height, leaf colour or shape, early or delayed flowering and a sterile panicle. These changes in phenotype were due mainly to somaclonal variations that occur during the transformation process, as evidenced by independent segregation of those phenotypes from the transgene. Such somaclonal variation-derived phenotypes were removed after four rounds of self-pollination, followed by selection of transgenic plants that grow normally compared with the NT controls. After such four rounds of prescreening, we were able to evaluate the effects of the transgene on agronomic traits using phenotypically homogeneous populations of the transgenic plants. Xiao *et al.* (2007, 2009) have previously reported a

Table 2 Up-regulated genes in RCc3:OsNAC5 and/or GOS2:OsNAC5 plants in comparison with nontransgenic controls

Gene	Loc No*	*RCc3:OsNAC5*		*GOS2:OsNAC5*		Stress response†
		Mean‡	*P*-value§	Mean‡	*P*-value§	
Genes up-regulated in both *RCc3:OsNAC5* and *GOS2:OsNAC5* plants						
Calcium-transporting ATPase	Os10g0418100	**10.36**	1.6E-04	**6.19**	6.3E-04	C
Cinnamoyl-CoA reductase	Os02g0811800	**8.55**	4.5E-06	**9.05**	3.9E-06	
Chitinase	Os11g0701500	**7.12**	9.9E-06	**14.20**	1.9E-06	
Cytochrome P450	Os12g0150200	**6.37**	2.1E-04	**4.79**	5.3E-04	C, D, S
CBS protein	Os02g0639300	**6.04**	3.1E-04	**3.70**	2.0E-03	
Sulfotransferase	Os01g0311600	**5.24**	5.0E-05	**7.60**	1.6E-05	
Aminotransferase	Os05g0244700	**5.18**	4.1E-06	**6.05**	2.4E-06	A, D, S
Chitinase	Os11g0701000	**4.97**	6.2E-06	**14.04**	4.2E-07	
Multicopper oxidase	Os01g0127000	**4.69**	9.8E-06	**4.91**	8.2E-06	
Nicotianamine synthase	Os07g0689600	**4.70**	2.8E-05	**5.15**	2.1E-05	
Pathogenesis-related transcriptional factor	Os07g0674800	**4.09**	3.0E-03	**12.03**	1.8E-04	
Cinnamoyl-CoA reductase	Os02g0808800	**4.14**	2.0E-05	**11.52**	9.7E-07	
Cinnamyl alcohol dehydrogenase	Os04g0612700	**3.90**	4.7E-04	**17.61**	1.0E-05	
ZIM	Os03g0180900	**4.06**	4.1E-05	**3.07**	1.3E-04	A, C, D, S
Glycoside hydrolase	Os05g0247800	**4.07**	4.4E-05	**4.04**	4.6E-05	A, S
Glutathione-*S*-transferase	Os10g0530500	**3.88**	5.1E-05	**4.81**	2.5E-05	
Iron-phytosiderophore transporter	Os02g0649900	**3.86**	5.7E-06	**5.40**	1.6E-06	
Aminotransferase	Os01g0729600	**3.21**	5.6E-04	**15.45**	5.9E-06	
Oxidase	Os06g0548200	**3.61**	2.6E-04	**3.82**	2.2E-04	
Disease resistance response protein	Os07g0643800	**3.07**	1.8E-04	**3.45**	1.0E-04	
WRKY	Os06g0649000	**3.39**	2.2E-04	**5.62**	3.9E-05	D, S
Acyltransferase	Os03g0245700	**3.06**	1.4E-04	**3.76**	5.5E-05	
Pyruvate kinase	Os04g0677300	**3.01**	4.0E-04	**3.66**	1.9E-04	
Oxidative stress response protein	Os03g0830500	**3.32**	2.3E-05	**4.07**	9.3E-06	D, S
9-cis-epoxycarotenoid dioxygenase	Os07g0154100	**3.53**	1.1E-02	**5.70**	3.0E-03	D, S
Genes up-regulated in *RCc3:OsNAC5* plants						
GLP	Os03g0693900	**32.65**	3.4E-06	1.05	4.3E-01	A, S
C4-dicarboxylate transporter	Os04g0574700	**30.10**	1.4E-06	1.11	1.9E-01	
***O*-methyltransferase**	Os10g0118200	**16.47**	1.8E-06	-1.46	2.9E-01	A, S
Fructose-bisphosphate aldolase	Os08g0120600	**11.27**	6.1E-06	1.01	1.8E-01	D, S
***O*-methyltransferase**	Os09g0344500	**8.43**	3.2E-05	-1.09	3.0E-01	A, S
MtN	Os05g0426000	**7.86**	7.2E-06	1.62	5.8E-02	
***O*-methyltransferase**	Os10g0118000	**7.09**	4.2E-05	-2.23	2.4E-02	S
Dehydration-responsive protein	Os11g0170900	**6.10**	9.8E-05	1.24	3.7E-02	D
Lipid transfer protein	Os01g0822900	**5.06**	8.4E-06	1.61	5.6E-03	
Oxidase	Os03g0693900	**4.86**	2.5E-04	1.99	5.6E-02	A, S
Glutamine synthetase	Os03g0712800	**4.16**	7.3E-05	1.25	1.2E-01	
Lipid transfer protein	Os11g0115400	**3.71**	4.8E-05	1.87	3.6E-03	A
PDX	Os07g0100200	**3.61**	3.1E-05	1.77	1.2E-03	
Cytochrome P450	Os01g0804400	**3.61**	1.8E-03	1.10	1.3E-01	
MERI5	Os04g0604300	**3.57**	6.0E-05	1.41	6.1E-03	
Homeobox	Os06g0317200	**3.33**	3.2E-04	−1.97	2.2E-03	
Pectin acetylesterase	Os01g0319000	**3.24**	5.0E-03	−1.50	7.5E-01	
bZIP	Os02g0191600	**3.20**	4.5E-03	−1.73	2.1E-01	
Lipid transfer protein	Os12g0115000	**3.08**	8.8E-04	1.76	4.8E-02	
Genes up-regulated in *GOS2:OsNAC5* plants						
Glutathione-*S*-transferase	Os09g0367700	1.30	1.9E-02	**10.26**	5.9E-06	A, D, S
Serine/threonine protein kinase	Os03g0269300	1.51	2.1E-03	**8.70**	9.0E-07	
WRKY	Os03g0335200	1.18	3.0E-02	**7.56**	5.2E-06	
Heavy metal transport/detoxification protein	Os04g0464100	1.20	5.9E-02	**6.78**	1.2E-05	
Stress response protein	Os01g0959100	−1.09	2.4E-01	**4.76**	3.1E-05	C, D, S
Auxin efflux carrier	Os08g0529000	1.19	2.9E-02	**4.52**	4.7E-06	
Subtilase	Os02g0270200	1.93	1.7E-03	**4.41**	2.2E-05	
UDP-glucuronosyl/UDP-glucosyltransferase	Os01g0638000	1.23	3.9E-02	**4.59**	5.3E-05	A, S
Disease resistance protein	Os06g0279900	−2.53	2.5E-03	**4.84**	7.2E-05	

Table 2 Continued

Gene	Loc No*	*RCc3:OsNAC5*		*GOS2:OsNAC5*		Stress response†
		Mean‡	*P*-value§	Mean‡	*P*-value§	
Nitrate reductase	Os02g0770800	−1.26	9.8E-01	**4.85**	8.7E-05	C
Heat shock protein	Os01g0606900	1.66	2.2E-03	**4.44**	2.3E-05	A, D, S
Phosphoenolpyruvate carboxykinase	Os10g0204400	1.28	9.4E-02	**3.29**	1.0E-04	
Xyloglucan endotransglycosylase	Os02g0280300	−2.13	1.1E-03	**3.95**	3.1E-05	
Isopenicillin N synthase	Os05g0560900	1.98	1.6E-04	**3.25**	8.8E-06	
Zinc finger	Os03g0820300	1.61	1.9E-03	**3.44**	3.8E-05	D, S
Serine/threonine protein kinase	Os09g0418000	1.60	1.8E-03	**3.07**	4.4E-05	A
ATPase	Os03g0584400	1.38	1.9E-02	**3.62**	4.3E-04	
Malic enzyme	Os05g0186300	1.88	4.9E-04	**3.06**	3.3E-05	

*Sequence identification numbers for the full-length cDNA sequences of the corresponding genes.

†Genes responsive to ABA (A), cold (C) drought (D) and salt (S) stress are based on microarray profiling data (accession number GSE31874).

‡The mean of two independent biological replicates. Numbers in boldface indicate up-regulation by more than three-fold ($P < 0.01$).

§*P* values were analysed by one-way ANOVA. Genes discussed in the text are in boldface. The microarray data sets can be found at http://www.ncbi.nlm.nih.gov/geo/ (Gene Expression Omnibus, accession number GSE31856).

significant reduction in grain yield under normal field conditions for transgenic rice plants harbouring exogenous stress-related genes when analysed at the T_1 and/or T_2 generation. These yield reductions would have been restored if the transgenic plants had been analysed at later generations after prescreening.

Yield components are sensitive to water stress at different stages of plant growth, such as anther dehiscence (Ekanayake *et al.*, 1989) and panicle exertion (O'Toole and Namuco, 1983). For example, drought stress at 12 days prior to anthesis can adversely affect spikelet fertility with severe reductions in grain yield (Cruz and O'Toole, 1984; Ekanayake *et al.*, 1989). The fact that the *RCc3:OsNAC5* plants had higher filling rates than the NT controls under drought conditions, reflects a reproductive stage tolerance to this condition. The delay in drought stress damage in the *RCc3:OsNAC5* plants might have allowed more spikelets to develop and flower normally. In contrast, the grain yield of the *GOS2:OsNAC5* plants under drought conditions was lower in 2009 and equivalent in 2010 and 2011 to the corresponding NT controls. The expression of *OsNAC5* in the whole plant body including the floral organs might have caused the reduction in the filling rate under drought conditions. Indeed, it has been shown that the use of constitutive promoters to express TFs s often causes unnecessary effects leading to unfavourable growth abnormalities (Hardy, 2010). For example, in previously reported analyses of *OsCc1:AP59* (Oh *et al.*, 2009) and *GOS2:OsNAC10* (Jeong *et al.*, 2010) plants, the floral organs were found to be significantly affected by the constitutive overexpression of the respective transgene.

The results of our JIP test in the present study, as manifested through the L-band of T_6 plants, showed that the energetic connectivity of antennas of PSII was present in all plants under drought conditions. This connectivity was highest in the *RCc3:OsNAC5* plants followed by the *GOS2:OsNAC5* plants when compared with the NT controls. Energetic connectivity is part of a protective mechanism that diverts excitation energies to photochemical pathways. This is similar to the photo-protective role of nonphotochemical quenching that diverts more excitation energy into heat dissipation (Horton *et al.*, 1996) because, at high-light flux, the excited chlorophylls in the core antennae of closed reaction centres (RCs) can potentially generate radicals leading to photoinhibition (Long *et al.*, 1994). Hence, if such connectivity was altered due to drought stress in our current transgenic plants, excess energy from PSII could have led to the production of reactive oxygen species (ROS) that ultimately cause photoinhibition. Hence, the results of our current JIP testing show that the *RCc3:OsNAC5* plants have more efficient and stable photosynthetic systems under drought conditions than the *GOS2:OsNAC5* plants and NT controls.

Roots are an important part of the plant architecture involved in foraging for water. With a deep and thick root system, plants can gain better access to water and show higher drought tolerance (Jeon *et al.*, 2011). This has been known for some time as O'Toole and Chang (1979) reported several decades ago that rice varieties with thicker roots were more tolerant to drought than those with thinner roots. The root diameter of the *RCc3:OsNAC5* plants was found in our analysis to be significantly larger than those of *GOS2:OsNAC5* and NT plants. The increase in root diameter of the *RCc3:OsNAC5* plants appeared to be caused by an enlargement of the metaxylem, a major part of the vascular bundle, and the aerenchyma, a tissue formed from cortical cell death. The vessel diameter has been demonstrated recently to be closely and positively correlated with better water flux and a lower risk of cavitation (Vasellati *et al.*, 2001; Yambao *et al.*, 1992). Zue *et al.* (2010) reported that the relative water content of mid-day leaf in the high root cortical aerenchyma lines are 10% greater than in the low root cortical aerenchyma lines under water stress. Taken together, our current findings and these previous results demonstrate that thickened roots are primarily responsible for increased tolerance leading to increased grain yield under drought conditions.

Our microarray experiments identified 19 and 18 root-expressed genes that were up-regulated specifically in the *RCc3:OsNAC5* and *GOS2:OsNAC5* plants, respectively, in addition to 25 root-expressed genes that were found to be up-regulated in both plants. These results were not surprising considering that the *RCc3* and *GOS2* promoters drive different patterns of transgene expression in roots. The *RCc3* promoter was active in the whole root tissues including vascular and cortex of the root elongation zone except for in root tip (apical meristem) region, whereas the *GOS2* promoter was active in root apical meristem and stele

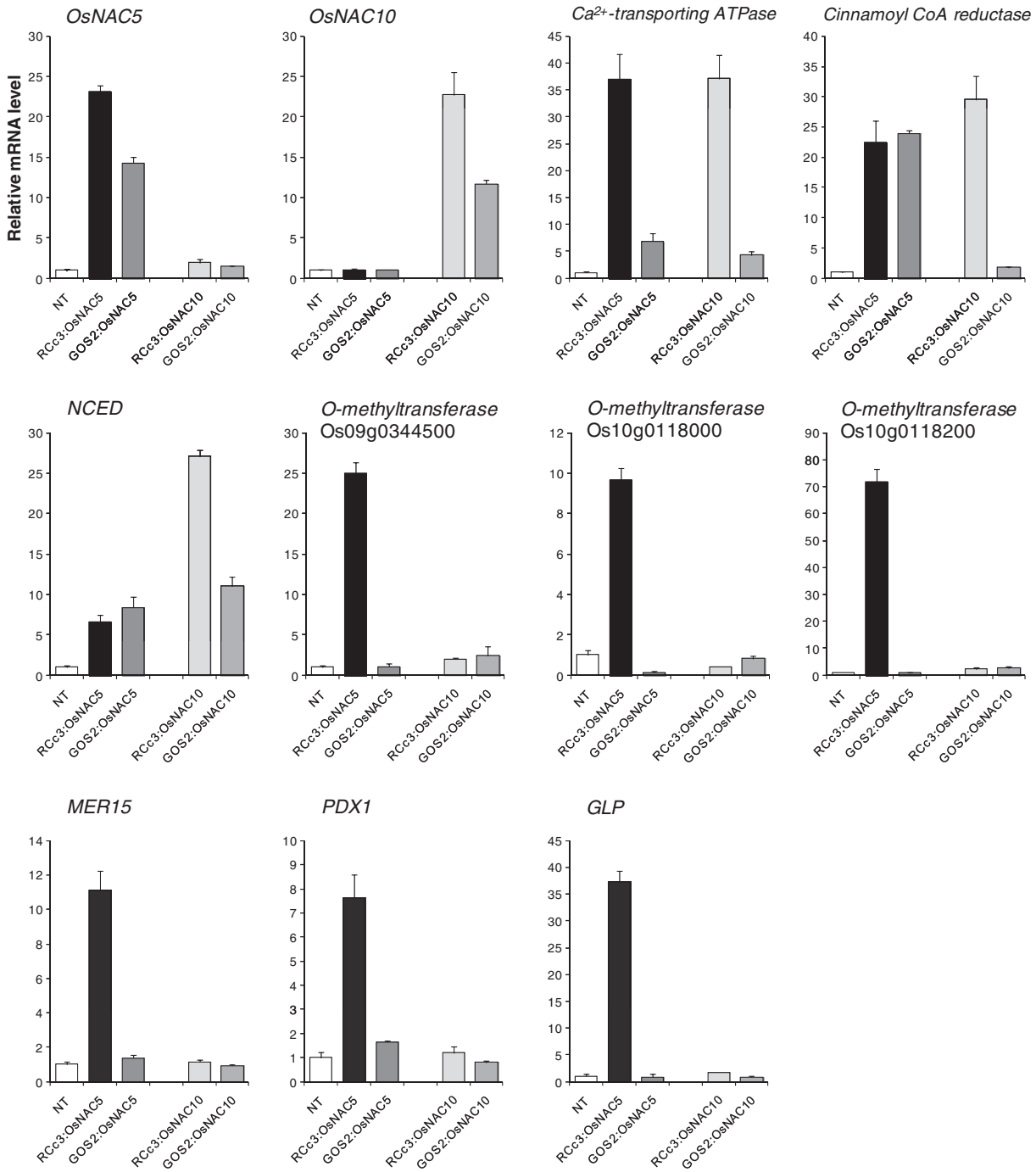

Figure 4 Regulated genes in roots of *OsNAC5* and *OsNAC10* plants under normal conditions. The transcript levels of *OsNAC5, OsNAC10* and eight target genes were determined by qRT-PCR (using the primers listed in Table S5), and each of transgenic rice plants is presented as a relative concentration to the levels in untreated nontransgenic (NT) control roots. Data were normalized using the rice ubiquitin gene (*OsUbi*) transcript levels. Values are the means ± SD of three independent experiments.

region of the root elongation zone (Figure S1). As the increase in root diameter of *RCc3:OsNAC5* plants resembles that of *RCc3:OsNAC10* plants that we previously reported (Jeong *et al.*, 2010), we compared expression patterns of *OsNAC5* target genes with those of the *OsNAC10* target genes. We found that only 17 of 62 *OsNAC5* target genes were up-regulated in *OsNAC10* roots (Figure 4, Table S4). In addition, expression specificities of those 17 genes were very different between the *OsNAC5* and *OsNAC10* roots, and the difference was confirmed by qPCR analysis (Figure 4). *GLP*, *PDX*, *MERI5* and *O-methyltransferases*, genes that are important for cell growth and development, were up-regulated in *RCc3:OsNAC5* roots, but neither in *RCc3:OsNAC10* nor in *GOS2:OsNAC10* roots. Taken together, *OsNAC5* up-regulates its target genes independently of *OsNAC10*, hence increases root diameter in a mechanism different from *OsNAC10*. Pyramiding of the two transgenes would strengthen tolerance of resultant plants to drought stress due mainly to their shared and distinct target genes.

Many of *OsNAC5* target genes are reported to function in stress responses, including *cytochrome P450*, *ZIM, oxidase, stress response protein* and *heat shock protein*. In particular, The *ZIM* is likely to be a direct target of *OsNAC5* because the rice *ZIM* promoter was shown to interact with the *TaNAC69* (Xue *et al.*, 2011), a homologue of *OsNAC5*. Also, identified in the roots of both transgenic plants were TFs such as WRKY, bZIP and zinc finger, in addition to ROS scavenging systems such as *multicopper oxidase*, *chitinase* and *glycosyl hydrolase*. The common genes that were up-regulated in both *RCc3:OsNAC5* and the *GOS2:OsNAC5* roots included *NCED, Calcium-transporting ATPase* and *Cinnamoyl CoA reductase*. The oxidative cleavage of *NCED* to generate xanthoxin is the critical and rate-limiting step in the regulation of ABA biosynthesis (Tan *et al.*, 1997). *AtNCED3* was detected exclusively in the vascular cell (Endo *et al.*, 2008), and its transgenic overexpressors improved drought tolerance (Iuchi *et al.*, 2001). *Calcium-transporting ATPase* is a major player in maintaining calcium homoeostasis in the cell. When cytosolic concentration of Ca^{2+} changed by influx of Ca^{2+} from outside the cell, or release of Ca^{2+} from internal store, *Calcium-transporting ATPase* serves as an early response to low temperature, drought and salinity stress in plant cells (Knight, 2000). *Cinnamoyl CoA reductase* is a key enzyme of the lignin biosynthesis pathway, controlling the quantity and quality of lignin (Jones *et al.*, 2001). Repression of the *AtCCR1* causes drastic phenotypic alterations (Goujon *et al.*, 2003). In addition, a loss-of-function mutation of this gene in maize (*Zmccr1*$^{-/-}$) results in a slight decrease in the lignin content and causes significant changes to the lignin structure (Tamasloukht *et al.*, 2011). The maize gene *ZmCCR2* has been found to be induced by drought conditions and can be detected mainly in roots (Fan *et al.*, 2006). Three of the target genes specifically up-regulated in *RCc3:OsNAC5* roots were *O-methyltransferases*, a gene encoding an enzyme involved in suberin biosynthesis. In Arabidopsis, transcripts of *ZRP4*, a gene which encodes an *O*-methyltransferase, have been reported previously to accumulate preferentially in the roots and localize predominantly in the endodermis region, with low levels also detectable in the leaves, stems and other shoot organs (Held *et al.*, 1993). The up-regulation of three *O-methyltransferase* genes in *RCc3:OsNAC5* roots may have contributed to the enhanced drought tolerance of the plants due to an increase in suberin biosynthesis. Lignin and suberin play major roles in impeding radial oxygen loss through lignification and/or suberization of the walls of the root peripheral layers in a process known as barrier formation. Lignin and suberin on the wall of endodermis and exodermis cells compose the casparian strip that inhibits the diffusion of water and solutes into stele (Takehisa *et al.*, 2012). Cai *et al.* (2011) have reported that the development of casparian strips on the endodermis and exodermis in salt- and drought-tolerant Liaohan 109 rice plants occurs at an earlier stage than the moderately salt-sensitive Tianfeng 202 or salt-sensitive Nipponbare strains. In maize, a close relative of rice, root developments are inhibited by severe drought stress due to cessation of root cell wall extension in elongation regions (Yamaguchi and Sharp, 2010). Lignifications were found to increase in drought stressed roots of maize, decreasing the extensibility of the cell wall. The increased lignifications of epidermis and xylem, in particular, were reported to restrict water loss from the root and also to facilitate longitudinal water transport in soybean (Yamaguchi and Sharp, 2010). *GLP*, *PDX* and *MERI5* that are known to function in cell growth and development were also found to be specifically up-regulated in *RCc3:OsNAC5* roots. Arabidopsis *GLP4*, which specifically binds to IAA, has been proposed to regulate cell growth (Yin *et al.*, 2009). *PDX* is involved in vitamin B6 biosynthesis, and Arabidopsis *pdx1.3* mutants show strongly reduced primary root growth and increased hypersensitivity to both salt and osmotic stress (Titiz *et al.*, 2006). The overexpression of *MERI5* in Arabidopsis leads to aberrant development with cell expansion alterations (Verica and Medford, 1997). Collectively, the increased expression of such target genes in *RCc3:OsNAC5* roots caused the enlargement of the root tissues thereby enhancing the tolerance to drought stress at the reproductive stages.

In summary, we here present the results of long-term field testing of transgenic rice overexpressing *OsNAC5* and the responses of these plants to drought stress. Importantly, we evaluated the agronomic traits of these transgenic crops at all stages of plant growth in the field. This allowed us to assess the advantages of using a regulatory gene such as *OsNAC5* to improve stress tolerance in a commercially important crop. Finally, we demonstrate from our results that the root-specific rather than whole-body expression of *OsNAC5* increases grain yield under drought conditions, indicating the potential use of this strategy for improving drought tolerance in other crops.

Experimental procedures

Plasmid construction and transformation of rice

The coding region of *OsNAC5* (AK102475) was amplified from rice total RNA using an RT-PCR system (Promega, Madison, WI) in accordance with the manufacturer's instructions. The primers used were forward (5′- ATGGAGTGCGGTGGTGCGCT-3′) and reverse (5′- TTAGAACGGCTTCTGCAGGT-3′). To enable the overexpression of *OsNAC5* in rice, the cDNA for this gene was linked to the *GOS2* promoter for constitutive expression and the *RCc3* promoter for root-specific expression using the Gateway system (Invitrogen, Carlsbad, CA; Figure S2). Plasmids were introduced into *Agrobacterium tumefaciens* LBA4404 by triparental mating, and embryogenic (*Oryza sativa* cv Nipponbare) calli from mature seeds were transformed as previously described (Park *et al.*, 2012b). The $T_{5–7}$ generations of single-copy independent lines were used for subsequent analysis.

RNA gel-blot analysis

Rice (*Oryza sativa* cv Nipponbare) seeds were germinated in soil and grown in a glasshouse (16-h-light/8-h-dark cycle) at 28 °C. For high-salinity and ABA treatments, 14-day-old seedlings were transferred to a nutrient solution containing 400 mM NaCl or 100 μM ABA, respectively, for the indicated periods in the glasshouse under continuous light of approximately 1000 μmol/m^2/s. For drought treatment, the 14-day-old seedlings were excised and air-dried for the indicated time course under continuous light of approximately 1000 μmol/m^2/s, as described previously (Redillas *et al.*, 2012). For low-temperature treatments, 14-day-old seedlings were placed in a 4 °C cold chamber for the indicated time course under continuous light of 150 μmol/m^2/s. The preparation of total RNA and RNA gel-blot analysis was performed as reported previously (Jung *et al.*, 2011). We repeated the experiments two times with two biological replicates.

Drought treatments of vegetative stage rice plants

Transgenic and NT rice (*Oryza sativa* cv Nipponbare) seeds were germinated in half-strength MS solid medium in a growth chamber in the dark at 28 °C for 4 days, transplanted into soil and then grown in a greenhouse (16-h-light/8-h-dark cycles) at

28–30 °C. Eighteen seedlings from each transgenic and non-transgenic (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 days followed by 7 days of watering. The numbers of plants that survived or continued to grow were then scored.

Chlorophyll fluorescence measurements

Transgenic and NT rice (*Oryza sativa* cv Nipponbare) seeds were germinated and grown in half-strength MS solid medium for 14 days in a growth chamber (16-h-light of 150 μmol/m^2/s/8-h-dark cycles at 28 °C). The green portions of approximately 10 seedlings were then cut using a scissors prior to stress treatments *in vitro*. All stress conditions were conducted under continuous light at 150 μmol/m^2/s. 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. F_v/F_m values were then measured as previously described (Oh *et al.*, 2008).

Rice 3′-tiling microarray

Expression profiling was conducted using the rice 3′-tiling microarray, manufactured by NimbleGen Inc. (http://www.nimblegen.com/) as previously described (Park *et al.*, 2012a). *RCc3:OsNAC5*-8, *GOS2:OsNAC5*-39 and NT rice (*Oryza sativa* cv Nipponbare) seeds were germinated in soil and grown in a glasshouse (16-h-light/8-h-dark cycle) at 22 °C. For the identification of genes up-regulated in *RCc3:OsNAC5*, *GOS2:OsNAC5* plants, total RNA (100 μg) was prepared from the root tissues of 14-d-old transgenic and NT rice seedlings (*Oryza sativa* cv Nipponbare) grown under normal conditions.

Evaluation of the agronomic traits of rice plants grown in the field

To evaluate the yield components of transgenic plants grown under normal field conditions, three independent T_5 (2009), T_6 (2010) and T_7 (2011) homozygous lines of the *RCc3:OsNAC5* and *GOS2:OsNAC5* plants, together with NT controls, were transplanted to a low land type paddy field at the Rural Development Administration, Suwon, Korea (2009) and the Kyungpook National University, Gunwi, Korea (2010 and 2011). A randomized design was employed with three replicates using three plots each with the size of 5 m^2 per plot. At 25 days after sowing, 22 seedlings per line were randomly transplanted within a 15 × 30 cm spacing and a single seedling type per hill. Fertilizer was applied at 70N/40P/70K kg/ha after the last paddling and 45 days after transplantation. Yield parameters were scored for 10 plants per plot for a total of 30 plants per line per season. Plants located at the borders were excluded from subsequent data scoring.

To evaluate the yield components of transgenic plants under drought field conditions, three independent T_5 (2009), T_6 (2010) and T_7 (2011) homozygous lines of each of the *RCc3:OsNAC5* and *GOS2:OsNAC5* plants, and NT controls, were transplanted into a 1-m-deep container filled with natural paddy soil covered by a removable rain-off shelter (located at Myongji University, Yongin, Korea). The experimental design, transplant spacing, use of fertilizer, drought treatments and scoring of agronomic traits were as described above for normal field conditions. The plants were exposed to drought stress at the panicle heading stage (from 10 days before heading to 10 days after heading). Following exposure to drought stress until complete leaf-rolling had occurred, plants were irrigated overnight and immediately subjected again to a second round of drought conditions until complete leaf-rolling again occurred. Upon completion of these drought treatments, plants were irrigated to allow recovery until the seed maturation stages. 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, Seoul, Korea), and weighed. The following agronomic traits were scored: panicle length, number of tillers, number of panicles, spikelets per panicle, filling rate (%) and total grain weight (g). The results from Fisher's least significance difference for multiple comparisons at $P < 0.05$ level under *post hoc* ANOVA and compared with the data from the NT controls. SPSS version 18.0 software was used to perform these statistical analyses.

Evaluation of root traits

To evaluate root phenotype, we used two events of the *RCc3:OsNAC5*-41 and -8 and the *GOS2:OsNAC5*-47 and -39 plants (see Figure 2a and Table S1). The transgenic and NT plants were transplanted to five PVC tubes (1.2 m in length and 0.2 m in diameter) contained with a low land paddy soil and placed in a 1.5-m-deep container located at Myongji University, Yongin, Korea. Only one seedling was transplanted per tube 25 days after sowing. Fertilizer was employed similarly as described for normal field conditions. Root observations were conducted before heading stage. PVC tubes were taken out from the container and removed the soil carefully. For each plant, only the longest root was used for measuring the length whilst the total roots were used for measuring the root volume per plant. For the root diameter, 10 roots per plant were measured, and the total roots per plant were used for the dry weight. SPSS version 18.0 was used for statistical analysis.

Microscopic examination of roots

The roots of transgenic and NT plants of 2 month old and the panicle heading stage were cut and washed two times with distilled H_2O. To dehydration, the samples were treated with graded ethanol series (30, 50, 70, 80, 95 and 100%) and three times in 100% ethanol each for 1 h. Dehydrated samples were further treated with a series of Technovit 7100 [30, 60, 80 and 100% (v/v) in EtOH] for 4 h each and then incubated in 100% Technovit solution for 1 day. The samples were solidified in plastic moulds with a mixture of Technovit and hardener solution II at room temperature for 2 days. Ultrathin sections (approximately 1 μm thick) were made using an ultramicrotome (MT-X; RMC Inc., Tucson, AZ) and observed and photographed under a light microscope.

JIP analysis

Chlorophyll *a* fluorescence transients in the plants were measured using the Handy PEA fluorimeter (Hansatech Instruments Ltd., King's Lynn, UK) as described previously (Redillas *et al.*, 2011a,b). Plants were dark-adapted for at least 30 min to ensure sufficient opening of the RCs, so that the RCs were fully oxidized. Two plants were chosen for each of the three independent T_6 homozygous lines. The tallest and the visually most healthy-looking leaves were selected from each plant and measured at

their apex, middle and base parts. The readings were averaged using the Handy PEA Software (version 1.31). The fluorimeter parameters were initial fluorescence at O (50 μs), J (2 ms) and I (30 ms) for intermediates, and P as the peak (500 ms–1 s). Transients were induced by red light at 650 nm of 3500 μmol photons m^2/s provided by the three light-emitting diodes, focused on a spot of 5 mm in diameter and recorded for 1 s with 12-bit resolution. Data acquisition was set at every 10 μs (from 10 μs to 0.3 ms), every 0.1 ms (from 0.2 to 3 ms), every 1 ms (from 3 to 30 ms), every 10 ms (from 30 to 300 ms) and every 100 ms (from 300 ms to 1 s). Normalizations and computations were performed using the Biolyzer 4HP software (v4.0.30.03.02) according to the equations of the JIP test. The difference kinetics at the OK phase (ΔW_{OK}) was calculated by subtracting the normalized data values for the stress-treated plants ($V_{OKsample}$) with the untreated NT plants ($V_{OKcontrol}$); $\Delta W_{OK} = V_{OKsample} - V_{OKcontrol}$. Normalization for each data set was performed using the equation $V_{OK} = (F_t - F_O)/(F_K - F_O)$. The results were analysed graphically using OriginPro 8 SR0 v9.0724 (Northampton, MA).

qPCR analysis

Total RNA was prepared as previously reported (Jung *et al.*, 2011). For quantitative real-time PCR experiments, a SuperScript™ III Platinum® One-Step Quantitative RT-PCR system (Invitrogen) was used. For PCRs, a master mix of reaction components was prepared, according to reported previously (Park *et al.*, 2012c), used Evagreen (SolGent, Seoul, Korea). Thermocycling and fluorescence detection were performed using a Stratagene Mx3000p Real-Time PCR machine (Stratagene, La Jolla, CA). PCR was performed at 95 °C for 10 min, followed by 40 cycles of at 94 °C for 30 s, 58 °C for 40 s and 68 °C for 1 min. To validate our qPCR results, we repeated each experiment three times. The primer pairs listed in Table S5.

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Supporting information

Additional Supporting information may be found in the online version of this article:

Figure S1 Expression pattern of *RCc3* and *GOS2* promoter in rice roots.
Figure S2 Schematic representation of rice transformation vectors.
Table S1 Agronomic traits of the *RCc3:OsNAC5* and *GOS2:OsNAC5* transgenic rice plants grown under normal field conditions.
Table S2 Agronomic traits of the *RCc3:OsNAC5* and *GOS2:OsNAC5* transgenic rice plants grown under drought conditions in the field.
Table S3 Agronomic traits of the nullizygous plants grown under normal field conditions.
Table S4 Comparison of *OsNAC5* target genes with *OsNAC10* target genes.
Table S5 Primers list for qPCR.

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