Plant Biotechnology Journal
Open Access

Plant Biotechnology Journal (2022) **20**, pp. 736–747 doi: 10.1111/pbi.13752

Transcriptional activation of rice *CINNAMOYL-CoA REDUCTASE 10* by OsNAC5, contributes to drought tolerance by modulating lignin accumulation in roots

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Received 15 February 2021;
revised 9 November 2021;
accepted 11 November 2021.
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Keywords: drought tolerance, *Oryza sativa*, *OsCCR10*, lignin biosynthesis.

Summary

Drought is a common abiotic stress for terrestrial plants and often affects crop development and yield. Recent studies have suggested that lignin plays a crucial role in plant drought tolerance; however, the underlying molecular mechanisms are still largely unknown. Here, we report that the rice (*Oryza sativa*) gene *CINNAMOYL-CoA REDUCTASE 10* (*OsCCR10*) is directly activated by the *OsNAC5* transcription factor, which mediates drought tolerance through regulating lignin accumulation. CCR is the first committed enzyme in the monolignol synthesis pathway, and the expression of 26 CCR genes was observed to be induced in rice roots under drought. Subcellular localisation assays revealed that OsCCR10 is a catalytically active enzyme that is localised in the cytoplasm. The *OsCCR10* transcript levels were found to increase in response to abiotic stresses, such as drought, high salinity, and abscisic acid (ABA), and transcripts were detected in roots at all developmental stages. *In vitro* enzyme activity and *in vivo* lignin composition assay suggested that OsCCR10 is involved in H- and G-lignin biosynthesis. Transgenic rice plants overexpressing *OsCCR10* showed improved drought tolerance at the vegetative stages of growth, as well as higher photosynthetic efficiency, lower water loss rates, and higher lignin content in roots compared to non-transgenic (NT) controls. In contrast, CRISPR/Cas9-mediated *OsCCR10* knock-out mutants exhibited reduced lignin accumulation in roots and less drought tolerance. Notably, transgenic rice plants with root-preferential overexpression of *OsCCR10* exhibited higher grain yield than NT controls plants under field drought conditions, indicating that lignin biosynthesis mediated by *OsCCR10* contributes to drought tolerance.

Introduction

Drought is often a major plant stress in land plants and can cause a severe loss of crop productivity (Pandey and Shukla, 2015). For example, in rice (*Oryza sativa*) drought can cause a reduction in spikelet numbers and low grain filling, critically affecting crop productivity. Therefore, there is considerable interest in better understanding the protective mechanisms that plants have evolved to avoid or tolerate with drought stress (Todaka *et al.*, 2015; Valliyodan and Nguyen, 2006). One such mechanism is accumulating secondary metabolites, including lignin (Liu *et al.*, 2015, 2020; Moura *et al.*, 2010; Tu *et al.*, 2020; Yoshimura *et al.*, 2008; Zhou *et al.*, 2020) and several studies have shown that drought tolerance can be affected by lignification. For example, overexpression of the transcription factor *PdNF-YB21* in poplar (*Populus*) promotes root growth along with enhanced lignification, leading to improved drought tolerance (Zhou *et al.*, 2020). Furthermore, overexpression of *VlbZIP30* in grapevine (*Vitis vinifera*) (Tu *et al.*, 2020), *PoCCoAOMT* in tobacco (*Nicotiana benthamiana*) (Zhao *et al.*, 2021), *IbLEA14* in sweet potato (*Ipomoea batatas*) (Park *et al.*, 2011), *OsTF1L* in rice (Bang *et al.*, 2019), and *PeLAC10*, *CmCAD2* and *CmCAD2* in *Arabidopsis thaliana* (Li *et al.*, 2020; Liu *et al.*, 2020) all enhances lignin biosynthesis and drought tolerance. In maize (*Zea mays*), drought-tolerant inbred lines showed higher lignification than drought-sensitive lines suggesting that lignification is an important adaptation to drought stress (Hu *et al.*, 2009).

Lignin is a complex polymer that is synthesised through the phenylpropanoid pathway, plays an important role in both biotic and abiotic stress responses (Liu *et al.*, 2018; Miedes *et al.*, 2014). It is composed of *p*-hydroxyphenyl (H), guiacyl (G) and sinapyl (S) units derived from monolignols which polymerise to form a secondary cell wall (Alejandro *et al.*, 2012; Bonawitz and Chapple, 2010; Miao and Liu, 2010). Lignin helps control water penetration through the cell wall thus and transpiration, maintaining the osmotic balance of the cell. Moreover, lignin deposition occurs in the walls of specific tissues to provide additional mechanical strength and water impermeability (Monties and Fukushima, 2005). Lignin biosynthesis occurs step wise and involves the formation of hydroxycinnamoyl-CoA esters, which are common precursors of monolignols (Dixon and Lamb, 1990; Hahlbrock and Scheel, 1989). Cinnamoyl-CoA reductase (CCR) catalyses the initial step of monolignol biosynthesis (Lauvergeat *et al.*, 2001), namely the conversion of coumaroyl-, feruloyl-, and sinapoyl-CoAs to coumaraldehyde, coniferaldehyde and sinapaldehyde, respectively (Davin *et al.*, 2008; Gross, 1981; Vanholme *et al.*, 2010).

A number of CCR homologs, including 11 in *A. thaliana* (Costa *et al.*, 2003), 9 in poplar (Shi *et al.*, 2010), and 26 in rice (Kawasaki *et al.*, 2006) have been identified. Biochemical characterisation and functional studies have shown that they are associated with different biological and developmental roles, such that *AtCCR1* is predominantly expressed in tissues undergoing lignification, while *AtCCR2* is expressed at very low levels during development (Lauvergeat *et al.*, 2001). Also, *AtCCR1* anti-sense plants shows a 50% decrease in lignin content (Goujon *et al.*, 2003) and over-expressing *CCR* can lead to increased accumulation of lignin (Goujon *et al.*, 2003; Lauvergeat *et al.*, 2001). However, the associated regulatory mechanisms remain mostly unknown.

In this study, we investigated the molecular functions and regulation of a CCR gene from rice, *OsCCR10*, in lignin biosynthesis and drought tolerance through the generation and characterisation of transgenic lines over-expressing *OsCCR10*, as well as CRISPR/Cas9-mediated *OsCCR10* knock-out mutants. The results are discussed in the context of a putative role for *OsCCR10* in lignin accumulation in roots and its consequent importance in drought tolerance.

Results

OsNAC5 directly regulates *OsCCR10* and enhance its expression

Previously, we characterised the transcription factor, *OsNAC5*, and showed that transgenic plants overexpressing *OsNAC5* had significantly greater drought tolerance than control plants (Jeong *et al.*, 2013). Expression profile analysis of *OsNAC5*-overexpressing plants led us to identify OsNAC5 as an enhancer of *OsCCR10* expression. Here, we examined the correlation between *OsNAC5* and *OsCCR10* expression through quantitative real time (qRT)-PCR analysis of plants constitutively expressing *OsNAC5* (*GOS2::OsNAC5*) and plants over-expressing *OsNAC5* specifically in roots (*RCc3::OsNAC5*) (Figure 1a). Notably, increases in *OsCCR10* transcript levels were higher in roots than in leaves in both *GOS2::OsNAC5* and *RCc3::OsNAC5* plants, suggesting that *OsNAC5* modulates the expression of *OsCCR10* predominantly in roots.

Cinnamoyl-CoA reductase catalyses the initial step in the monolignol pathway for lignin biosynthesis (Figure S1a). To investigate a potential connection between *OsCCR10* expression and lignin biosynthesis, we examined lignin accumulation in 2-month-old non-transgenic (NT), *GOS2::OsNAC5*, and *RCc3::OsNAC5* plants (Figure 1b). Lignin levels were higher in roots of *GOS2::OsNAC5* and *RCc3::OsNAC5* plants than in the NT control plants, while levels in leaves and stems of *GOS2::OsNAC5*, *RCc3::OsNAC5*, and NT plants remained unchanged. These observations led us to hypothesise that *OsCCR10* modulates lignification in the roots of *OsNAC5* over-expressing plants.

To confirm whether *OsCCR10* is a direct target of OsNAC5, we performed chromatin immunoprecipitation (ChIP) coupled with qRT-PCR analysis using *PGD1-Myc*^{OsNAC5} (myc-tagged *OsNAC5* overexpressor) and NT plants (Figure 1c and Figure S2). ChIP-PCR analysis showed that OsNAC5 was significantly enriched on the *OsCCR10* promoter, indicating that OsNAC5 likely regulates *OsCCR10* expression. We further validated the transcriptional activation of *OsCCR10* by OsNAC5 using a transactivation assay (Figure 1d). The *OsCCR10* promoter was fused to a firefly luciferase (fLUC) reporter and co-transfected into rice protoplasts with either a *35S::OsNAC5* construct or an empty vector. The expression of the *OsCCR10* reporter was significantly elevated in the presence of the *35S::OsNAC5* construct but not in the presence of the empty vector, suggesting that OsNAC5 binds to and activates *OsCCR10*.

Molecular characterisation of *OsCCR10*

To identify the rice *CCR* genes (Figure S1b) involved in drought tolerance responses, we examined their expression patterns by qRT-PCR when the roots were exposed to drought conditions (Figure S1c,d). All of the known 26 *OsCCR* genes were induced in roots under drought conditions, consistent with a role in the protective mechanisms against drought.

OsCCR10 was chosen for further functional characterisation of *OsNAC5*-mediated drought-tolerance, since its expression was predominantly induced in roots under drought and high salinity conditions and in response to abscisic acid (ABA) treatment, but showed no significant induction by low-temperature treatments (Figure 2a). We next investigated *OsCCR10* transcript levels in various tissues at different developmental stages (Figure 2b). *OsCCR10* expression was highest in roots at all developmental stages, with particularly high levels in the late 60 days stage. In summary, *OsCCR10* is highly expressed in roots and its expression is induced by drought, high salinity and ABA.

To determine the subcellular localisation of OsCCR10, we generated a construct with its coding sequence (CDS) translationally fused with a green fluorescent protein (GFP) and expressed it in rice protoplasts under the control of the *35S* promoter (*35S::OsCCR10-GFP*; Figure 2c). Peroxisome, Golgi, and endoplasmic reticulum (ER) markers were used to visualize subcellular organelles (Nelson *et al.*, 2007). The GFP signal was abundant in the cytosol and no obvious overlap was detected with the tested subcellular organelle markers, indicating that OsCCR10 is a cytosolic protein.

Next, the activity of recombinant OsCCR10 was assayed using *in vitro* assays with coumaroyl-, feruloyl- and sinapoyl-CoA substrates, which represent precursors for the H-, G- and S- units of lignin, respectively (Figure 3). Since NADPH is a cofactor for the reduction of hydroxycinnamoyl-CoA by CCR, a decrease in the levels of NADPH provides a measure of CCR activity (Park *et al.*, 2017). When the hydroxycinnamoyl-CoA substrate (30 μM) and purified recombinant OsCCR10 protein (16 or 32 μg) were used, OsCCR10 was observed to have reductase activity with coumaroyl-CoA and feruloyl-CoA, but not with sinapoyl-CoA. These results suggest that OsCCR10 is involved in H- and G-lignin biosynthesis.

Overexpression of *OsCCR10* significantly increases lignin accumulation in roots

To investigate the biological functions of *OsCCR10*, we generated two different types of transgenic rice plants: one over-expressing *OsCCR10* throughout the plant (*GOS2::OsCCR10*) and specifically over-expressing *OsCCR10* in roots (*RCc3::OsCCR10*) (Figure S3a). From 30 individuals of each type we selected homozygous T_3 transgenic lines, from which somaclonal variations had been eliminated by successive field selection. We then selected three independent homozygous lines for each of *GOS2::OsCCR10* (#8, 9 and 13) and *RCc3::OsCCR10* (#2, 16 and 27) for further study.

The expression levels of *OsCCR10* were elevated in both leaves and roots of *GOS2::OsCCR10* plants, while *OsCCR10* expression increased only in roots of *RCc3::OsCCR10* plants (Figure 4a,b). To confirm that the lignin accumulation in the roots of *GOS2::*

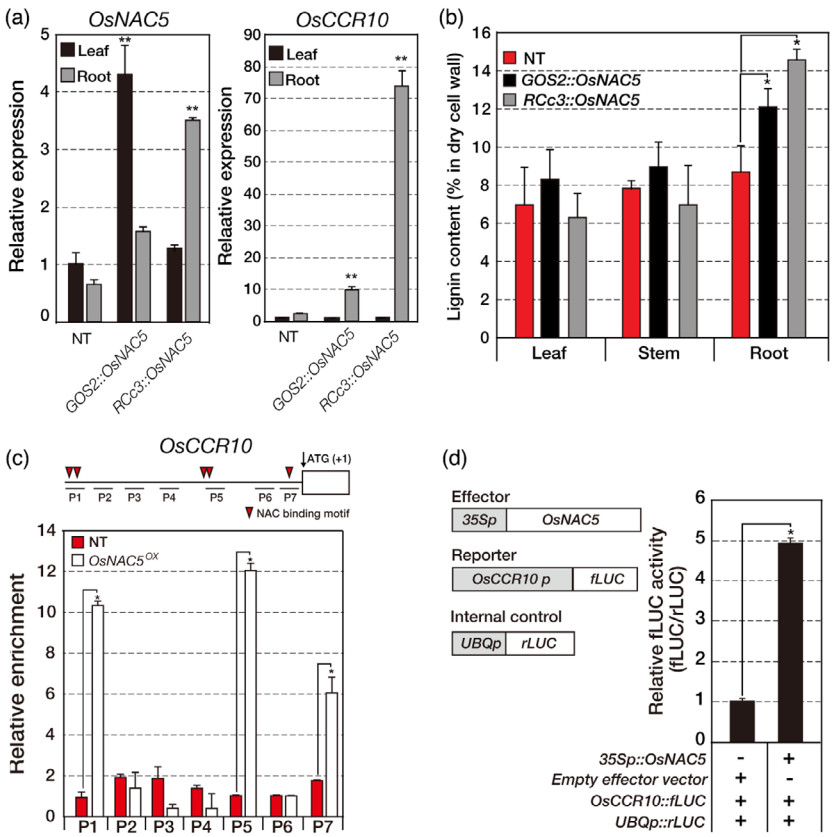

Figure 1 OsNAC5 directly enhances the *OsCCR10* expression. (a) Relative expression of *OsNAC5* and *OsCCR10* in *GOS2::OsNAC5* and *RCc3::OsNAC5* tissues. *OsUbi1* (AK121590) expression was used as an internal control. Data bars represent the mean ± SD of two biological replicates, each with three technical replicates. Data are shown as the mean ± SD of three independent experiments. Asterisks indicate significant differences compared with NT (**$P < 0.01$, Student's *t*-test). (b) Lignin content in 2-month-old *GOS2::OsNAC5*, *RCc3::OsNAC5* and NT rice plants. Data bars represent the mean ± SD of three biological replicates ($n = 3$), each with two technical replicates. Asterisks indicate significant differences compared with NT (*$P < 0.05$, one-way ANOVA). (c) Two-week-old *GOS2::OsNAC5-myc* and NT plants were used in a ChIP-qPCR experiments with an anti-myc antibody. Promoter regions showing three PCR-amplified regions (P1–P7). ChIP-qPCR data show PCR amplified regions of each gene. The relative enrichment was normalised to total input. Data are shown as the mean ± SD of three independent experiments. (d) Transient protoplast expression assay using a dual-luciferase reporter system. Schematic diagram of the effector, internal control and reporter constructs. Relative fLUC (fLUC/rLUC) activity in rice protoplasts. Data are shown as the mean ± SD of three independent experiments. Asterisks indicate significant differences compared with NT (*$P < 0.05$, Student's *t*-test).

OsNAC5 and *RCc3::OsNAC5* plants was caused by up-regulation of the *OsCCR10* target gene, we measured lignin levels in *GOS2::OsCCR10*, *RCc3::OsCCR10* and NT plants. Lignin levels were significantly higher in the roots of *GOS2::OsCCR10* and *RCc3::OsCCR10* plants than in NT plants. Interestingly, lignin levels in leaves and stems of *GOS2::OsCCR10*, *RCc3::OsCCR10* and NT plants were similar (Figure 4c). The same was true for leaves and stems of *GOS2::OsNAC5* and *RCc3::OsNAC5* plants (Figure 1b). Moreover, lignin-specific (phloroglucinol-HCl) staining showed the same pattern of lignin accumulation in the *GOS2::OsCCR10* and *RCc3::OsCCR10* plants (Figure 4d). In particular, the sclerenchyma tissue, as well as the vasculature tissues in *GOS2::OsCCR10* and *RCc3::OsCCR10* roots, were highly lignified, whereas the corresponding stem tissues in *GOS2::OsCCR10* and *RCc3::OsCCR10* plants was less lignified (Figure 4e).

Further, we analysed the lignin composition of *RCc3::OsCCR10* roots via an analytical pyrolysis coupled to gas chromatography with mass-spectrometer. The results showed that quantity of total aromatic components in the transgenic roots was 40%–80% higher than that of NT roots (Table 1). Moreover, several G-lignin components, *o*-Cresol, 2,4-Xylenol and Guaiacol, 4-ethyl-, were detected in transgenic roots but not in NT roots (Table 1). H and G lignin contents of transgenic roots was 45% higher than that of NT roots (Table 2), which suggests that CCR10 is involved in H and G lignin biosynthesis. Taken together, our results demonstrate that *OsCCR10* enhances lignification of roots through its reductase activity to the coumaroyl-CoA and feruloyl-CoA, consequently increasing H and G lignin, respectively.

Overexpression of *OsCCR10* confers drought stress tolerance

Since the *OsNAC5* over-expressing plants had a drought-tolerant phenotype, we tested the drought tolerance of *GOS2::OsCCR10* and *RCc3::OsCCR10* plants after drought treatments. Six-week-old plants were exposed to drought conditions for 3 days by withholding water (Figure 5a), resulting in the soil moisture content that decreased at a constant rate over time, reaching ~10% 3 days after the initiation of the drought treatment (Figure 5b). Visual symptoms of drought-induced damage, such as leaf rolling and wilting, were delayed in *GOS2::OsCCR10* and *RCc3::OsCCR10* plants compared to NT plants. After re-watering,

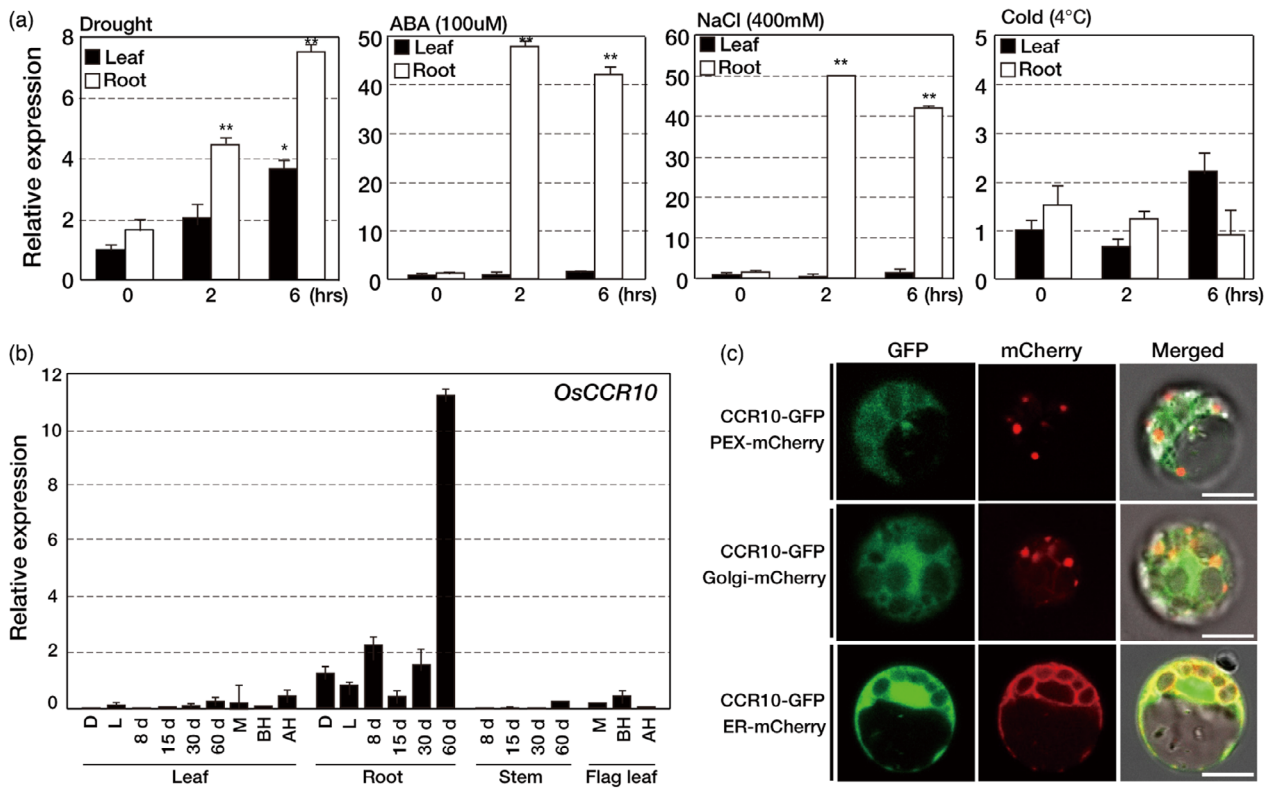

Figure 2 *OsCCR10* expression and subcellular localisation of the corresponding protein. (a) Relative expression of *OsCCR10* in response to abiotic stresses. Two-week-old seedlings were exposed to air-drying (drought), 100 μM ABA, 400 mM NaCl and at 4 °C (cold) for the indicated times. *OsUbi1* expression was used as an internal control. Values are the means ± SD of three independent experiments. Asterisks indicate significant differences compared with NT (*$P < 0.05$, **$P < 0.01$; Student's *t*-test) (b) Quantitative real time (qRT)-PCR of *OsCCR10* in various tissues and at different growth stages. (D, dark; L, light; d, day after germination; M, <1 cm in panicle length; BH, before heading; AH, after heading). *OsUbi1* expression was used as an internal control. Data bars represent the mean ± SD of two biological replicates with three technical replicates in each. (c) The construct of OsCCR10 fused to GFP (*35S::OsCCR10-GFP*) was transiently expressed in the rice protoplast. *PEX-mCherry*, *Golgi-mCherry*, and *ER-mCherry* were the controls for localisation in peroxisomes, Golgi, and ER, respectively (Nelson *et al.*, 2007). Scale bar, 10 μm.

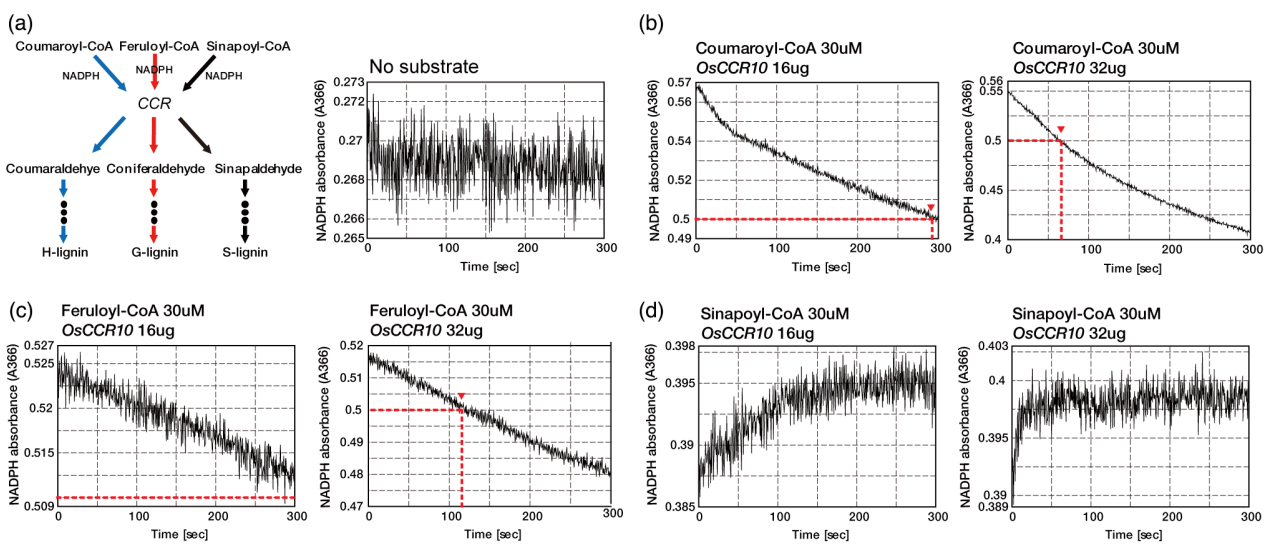

Figure 3 Enzymatic activity of recombinant OsCCR10. (a) Schematic diagram of OsCCR10 activity. A366, representing changes in NADPH levels, was monitored for 5 min. Changes in measured A366 values were monitored with NADPH as a negative control. (b–d) OsCCR10 activity was determined using coumaroyl-CoA, feruloyl-CoA and sinapoyl-CoA as substrates. Two different concentrations of OsCCR10 were tested to determine reaction kinetics.

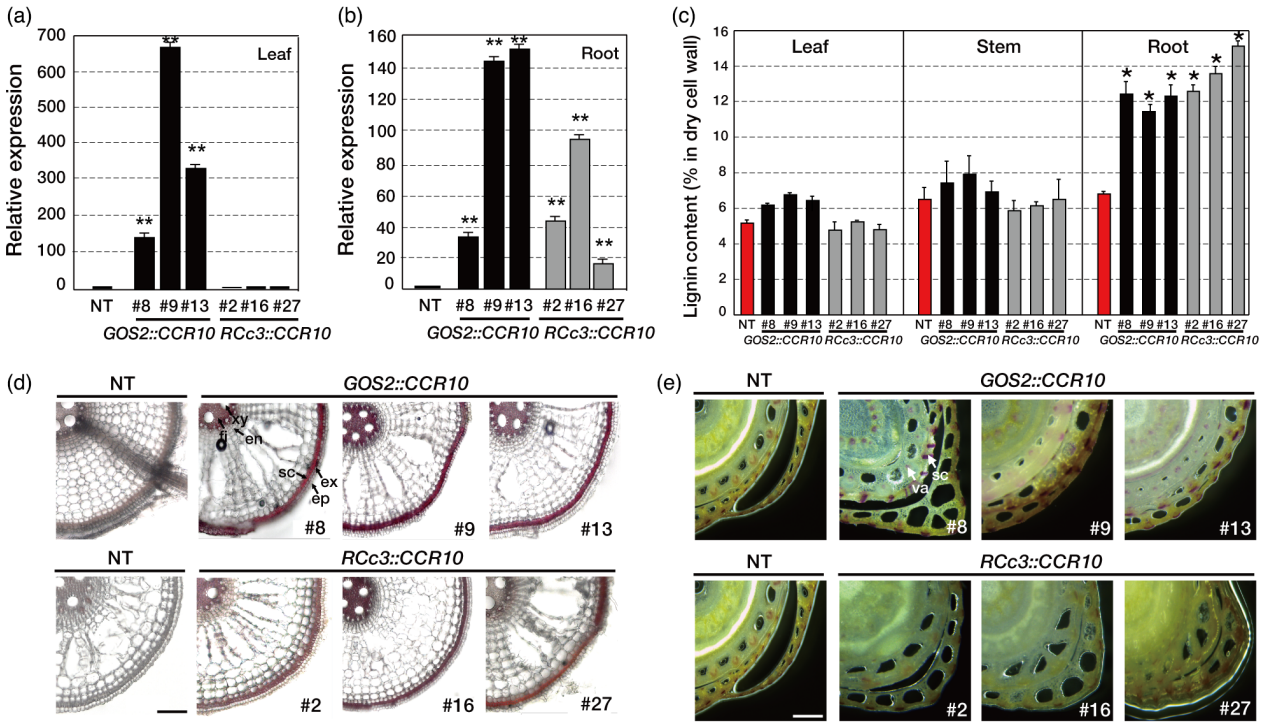

Figure 4 Accumulation of lignin in *GOS2::OsCCR10* and *RCc3::OsCCR10* transgenic rice and control plants. (a, b) Quantitative real time (qRT)-PCR analysis of *OsCCR10* expression in 2-week-old *GOS2::OsCCR10* (#8, 9, 13), *RCc3::OsCCR10* (#2, 16, 27) and NT plants. *OsUbi1* was used as an internal control. Data bars represent the mean ± SD of two biological replicates, each with three technical replicates. (c) Lignin contents in 2-month-old *GOS2::OsCCR10*, *RCc3::OsCCR10* and NT rice plants. Data bars represent the mean ± SD of three biological replicates (n = 3), each with two technical replicates. Asterisks indicate significant differences compared with NT ($P < 0.05$, one-way ANOVA). (d) Transverse sections of phloroglucinol-HCl stained 2-month-old NT, *GOS2::OsCCR10* and *RCc3::OsCCR10* roots. Black arrowheads points to root cellular structures stained by phloroglucinol-HCl. en, endodermis; me, metaxylem; fi, fibre; sc, sclerenchyma; ex, exodermis; ep, epidermis. (e) Transverse hand sections of phloroglucinol-HCl stained 2-month-old NT, *GOS2::OsCCR10* and *RCc3::OsCCR10* mature stems. White arrowheads indicate cellular structures in stems stained with phloroglucinol-HCl. va, vascular bundles; sc, sclerenchyma tissue.

Table 1 Quantitative analysis of aromatic component from root samples (μg/mg sample)

No.	RT (min)	Component	Unit	NT (SD)	*RCc3::OsCCR10* #16 (SD)	*RCc3::OsCCR10* #27 (SD)
1	4.06	Benzene		3.8 (0.31)	7.0* (0.04)	4.7* (0.32)
2	6.24	Toluene		1.4 (0.12)	5.2* (0.22)	3.3* (0.13)
3	10.04	Xylene		1.1 (0.20)	1.6* (0.20)	1.2 (0.20)
4	10.87	Styrene		0.3 (0.02)	0.8* (0.10)	0.6* (0.10)
5	15.66	Phenol	H	0.9 (0.10)	1.0 (0.01)	1.4* (0.11)
6	19.27	*o*-Cresol	G	–	0.2* (0.01)	0.2* (0.01)
7	20.51	*p*-Cresol	H	0.4 (0.02)	0.5 (0.02)	0.5 (0.01)
8	20.92	Guaiacol	G	1.2 (0.11)	2.3* (0.13)	2.0* (0.10)
9	23.97	2,4-Xylenol	G	–	0.4* (0.01)	0.5* (0.10)
10	24.94	Phenol, 4-ethyl-	H	0.5 (0.01)	0.6* (0.03)	0.7* (0.10)
11	25.99	Creosol	G	0.1 (0.01)	0.4* (0.01)	0.2 (0.01)
12	27.65	4-vinyl-phenol	H	0.4 (0.02)	0.2 (0.01)	0.3 (0.01)
13	29.81	Guaiacol, 4-ethyl-	G	–	0.2* (0.01)	0.2* (0.01)
14	31.64	Vinylguaiacol	G	5.2 (0.22)	6.8* (0.42)	5.8 (0.41)
15	33.35	Syringol	S	–	–	–
Total				15.2 (1.14)	27.3* (1.22)	21.6*(1.64)

SD, standard deviation.

Asterisks indicate significant differences compared with NT (*$P < 0.05$, Student's t-test).

Table 2 Summary of lignin derived components in group from root samples (μg/mg sample)

Sample	H unit (SD)	G unit (SD)	S unit	S/G	H:G:S
NT	2.2 (0.14)	6.5 (0.34)	–	–	26:74:0
RCc3::OsCCR10 #16	2.2 (0.04)	10.4* (0.46)	–	–	18:82:0
RCc3::OsCCR10 #27	3.0* (0.23)	9.3* (0.64)	–	–	25:75:0

SD, standard deviation.

Asterisks indicate significant differences compared with NT (*$P < 0.05$, Student's *t*-test).

the *GOS2::OsCCR10* and *RCc3::OsCCR10* plants rapidly recovered, whereas the NT plants did not (Figure 5a). Furthermore, the *GOS2::OsCCR10* and *RCc3::OsCCR10* plants showed a higher recovery rate than the NT plants after re-watering (Figure 5c). To verify the drought tolerance, Fv/Fm values, which were used as indicators of photochemical efficiency, were measured under drought conditions (Figure 5d). In NT plants, the values started to decrease 1 day after initial exposure to drought stress, whereas in *GOS2::OsCCR10* and *RCc3::OsCCR10* plants the values decreased slightly only after 3 days of exposure to drought stress. Collectively, these results suggested that *OsCCR10* over-expression confers drought tolerance during the vegetative stage of growth.

Since the productivity of rice plants is seriously affected by drought stress, we evaluated yield parameters of *GOS2::OsCCR10* and *RCc3::OsCCR10* plants grown in a paddy field under normal and drought conditions. Three independent *GOS2::OsCCR10* and *RCc3::OsCCR10* T_3 lines and NT plants were planted in a paddy field. Yield parameters were scored for 20 plants per independent line with two replicates (Figure 5e and Table S2). All of the yield parameters, including the filling rate (FR) and total grain weight (TGW), were similar in the transgenic and NT plants under normal field conditions, indicating that overexpression of *OsCCR10* does not affect grain productivity under normal growth conditions. However, under drought conditions, the FR and TGW values were significantly higher in the *RCc3::OsCCR10* plants, while they were similar in *GOS2::OsCCR10* and NT plants. These results suggested that root-preferential overexpression of *OsCCR10* enhanced drought tolerance of plants at the reproductive stage, resulting in greater grain yield under drought conditions compared to NT controls.

Finally, we examined the water loss rate in *GOS2::OsCCR10*, *RCc3::OsCCR10* and NT plants under drought conditions (Figure 5f). The water loss of *GOS2::OsCCR10* and *RCc3::OsCCR10* plants was slower than in NT plants under drought conditions.

OsCCR10 knockout lines show increase drought stress susceptibility

To test whether *OsCCR10* is necessary for the observed drought response, we generated CRIPSR/Cas9^{OsCCR10} knock-out plants (Figure S3b). The CRIPSR/Cas9^{OsCCR10} mutants contained a biallelic mutation comprising a two base deletion (line #2) or a one base insertion (lines #8 and 9), (Figure 6a). To investigate the effect of the *OsCCR10* mutation on drought response in rice plants, we subjected CRIPSR/Cas9^{OsCCR10} and NT plants to drought stress, before monitoring the drought-induced symptoms (Figure 6b). Soil moisture content was measured and a similar rate of decrease indicated that stress treatments were uniformly applied across the plants (Figure 6c). Drought-induced symptoms, such as leaf rolling and wilting, appeared earlier in CRIPSR/Cas9^{OsCCR10} lines compared to NT control plants in response to the drought stress treatments, and NT plants showed a higher recovery rate than CRIPSR/Cas9^{OsCCR10} plants after re-watering. NT plants had a 75%–95% survival rate 4 days after re-watering, while only 3%–20% of the CRIPSR/Cas9^{OsCCR10} plants survived (Figure 6d). Lignin levels were significantly lower in CRIPSR/Cas9^{OsCCR10} roots than in NT roots, while lignin levels in leaves and stems were similar in mutant and NT plants (Figure 6e).

To further elucidate the contribution of lignin to the drought stress response in rice, we examined lignin accumulation in wild-type plants as a consequence of the drought treatments (Figure S4). Six-week-old plants were exposed to drought conditions for 3 days by withholding irrigation. After the drought stress treatments, lignin levels in the stems and roots increased, whereas they remained unchanged in the leaves, which suggests that lignin accumulation in the roots plays a key role in drought tolerance.

Discussion

Previously, we identified that a NAC transcription factor, OsNAC5, is a positive regulator of drought tolerance in rice plants (Jeong *et al.*, 2013). Transcriptome analysis of *OsNAC5* overexpressing plants showed that CCR genes, including *OsCCR1* (Os02g0808800) and *OsCCR10* (Os02g0811800), were up-regulated, being potential target genes of OsNAC5 (Jeong *et al.*, 2013). Here, we confirm that the expression of *OsCCR10* is directly regulated by *OsNAC5* and the accumulation of lignin was substantially higher in *OsNAC5*-overexpressing roots than in NT roots (Figure 1). Genome-wide transcriptome analysis of multiple *OsNAC* transgenic plants (*OsNAC5*, *6*, *9*, and *10*) that were drought-tolerant, revealed that expression level of *OsCCR10* was significantly higher in *OsNAC5* over-expressing plants than the others (Chung *et al.*, 2018). In addition, *OsNAC5* is particularly associated with the modification of root architecture and lignin composition (Chung *et al.*, 2018; Jeong *et al.*, 2013).

Many studies have shown that CCR genes are essential for lignin biosynthesis (Goujon *et al.*, 2003; Lauvergeat *et al.*, 2001; Park *et al.*, 2017); however, their physiological roles in response to abiotic stress have not yet been fully elucidated. Recent reports have revealed that lignin contributes to plant drought tolerance. For example, in grapevine, over-expression of *VlbZIP30* led to high lignification levels in leaves, thereby enhancing drought tolerance (Tu *et al.*, 2020). Additionally, overexpression of *OsERF71* in rice was reported to enhance drought tolerance due to increased lignin accumulation in roots (Lee *et al.*, 2016), and it was reported that lignin accumulation and an increase in *CCR* expression are drought stress responses in plants (Liu *et al.*, 2018; Xie *et al.*, 2018). However, direct genetic evidence for CCR function in drought tolerance has not been reported so far.

The lignin biosynthetic pathway can be divided into three phases involving monolignols: (i) biosynthesis; (ii) transport; and (iii) polymerisation (Figure S1a). The monolignols are synthesised in the cytoplasm and transported across the plasma membrane to the secondary cell wall where they are polymerised to form the deposits of polymeric lignins (Liu *et al.*, 2018). Our analysis of subcellular localisation indicated that OsCCR10 accumulates in the cytoplasm (Figure 2c). And *in vitro* enzyme assays and *in vivo* lignin composition assays showed that OsCCR10 catalyses H-,

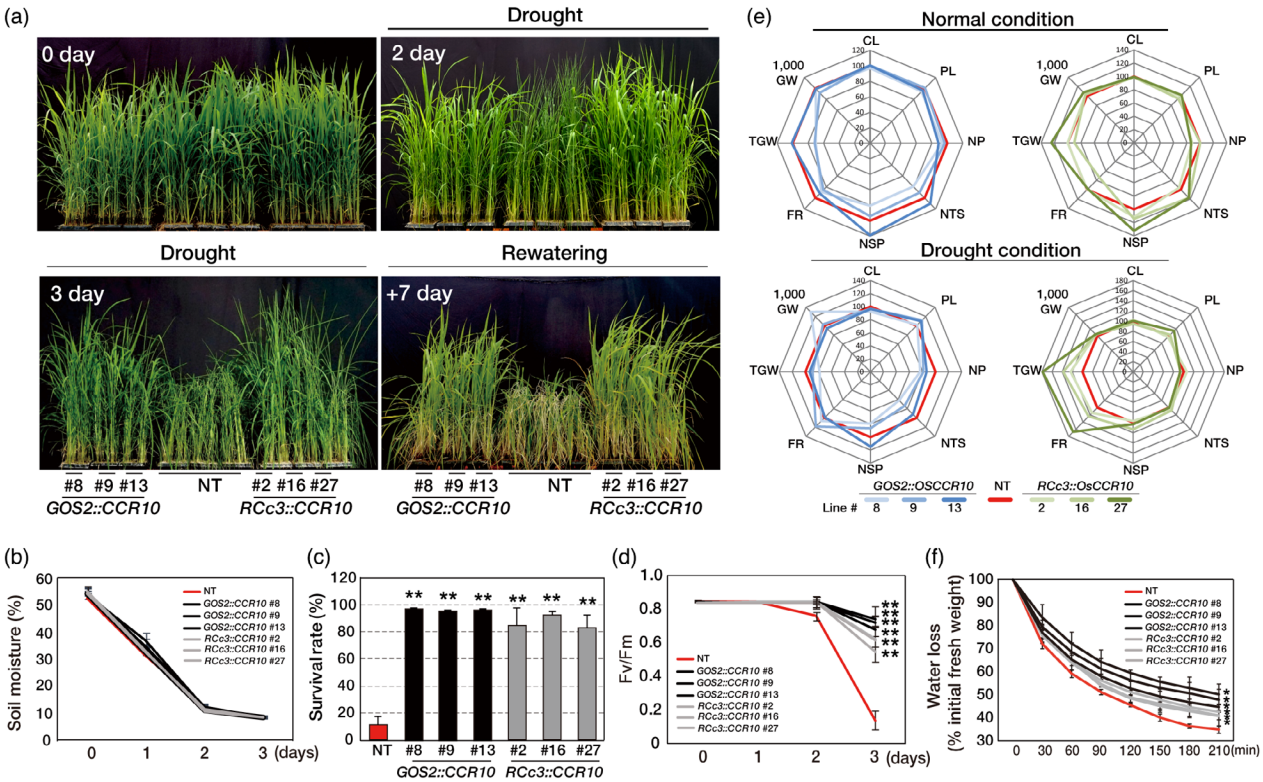

Figure 5 Drought tolerance of *GOS2::OsCCR10* and *RCc3::OsCCR10* plants. (a) Phenotypes at the vegetative stage of *GOS2::OsCCR10* and *RCc3::OsCCR10* transgenic rice plants grown under drought stress. Three independent homozygous *GOS2::OsCCR10* and *RCc3::OsCCR10* T_3 lines and NT control plants were grown in soil for 6 weeks and exposed to drought for 3 days, followed by re-watering. (b) Soil moisture in the pots exposed to drought treatment at the indicated time points. Values are the means ± SD (n = 10). (c) Survival rate scored 7 days after re-watering. Values are the means ± SD (n = 30). Asterisks indicate significant differences compared with NT (*P < 0.05, **P < 0.01; one-way ANOVA) (d) Chlorophyll fluorescence (Fv/Fm) of *GOS2::OsCCR10* and *RCc3::OsCCR10* and NT rice plants during 3 days of drought treatment. Fv/Fm values were measured in the dark to ensure sufficient dark adaptation. Data are shown as the mean ± SD (n = 30). Asterisks indicate significant differences compared with NT (**P < 0.01, one-way ANOVA) (e) Agronomic traits of *GOS2::OsCCR10* and *RCc3::OsCCR10* transgenic rice plants grown in the field under both normal and drought conditions. Spider plots of yield parameters for three independent homozygous T_3 lines of *GOS2::OsCCR10* and *RCc3::OsCCR10* under normal and drought conditions, respectively. Each data point shows a percentage of the mean values (n = 20) listed in Table S2. Mean values from NT controls were set at 100% as a reference. CL, culm length; PL, panicle length; NP, number of panicles per hill; NTS, number of total spikelets; NSP, number of spikelets per panicle; FR, filling rate; TGW; total grain weight; 1000 GW, weight of 1000 grains. (f) The relative water content of detached leaves. For each replicate, 3-month-old mature plants were used. Data are shown as the mean ± SD of three independent lines and two experimental replicates. Asterisks indicate significant differences compared with NT (*P < 0.05, one-way ANOVA).

and G-monolignol synthesis (Figure 3 and Table 1), which support OsCCR10 being involved in lignin biosynthesis in roots.

In this study, lignin accumulation was higher in the sclerenchyma and root fibres in both whole body over-expressing (*GOS2::OsCCR10*) and root-specific over-expressing (*RCc3::OsCCR10*) plants, whereas it was lower in roots of the CRIPSR/Cas9^{OsCCR10} plants (Figures 4c,d, and 6e). Thus, lignin accumulation was increased only in root tissues even when *OsCCR10* was over-expressing in a whole plant. The same patterns of root-preferential lignin accumulation were observed in *GOS2::OsNAC5* plants (Figure 1b). This root-preferential lignin accumulation might be due to both substrate availability and/or the expression pattern of enzymes involved in the lignin biosynthesis pathway (Barros *et al.*, 2015; Meyer *et al.*, 1998). For example, secondary cell walls of tracheary elements, a key component of xylem tissue, is mainly composed of G-units, and the Casparian strip is composed of a mixture of G- and S-units in monocots. Key enzymes involved in lignin monomer synthesis, such as C4H, 4CL, CCoAOMT and CAD, show cell-specific expression patterns (Barros *et al.*, 2015). These reports suggest that CCR enzymes may have different physiological roles in different tissues. Our results support that *OsCCR10* plays a specific role in roots for drought tolerance.

It has been proposed that lignin plays a barrier in maintaining cell osmotic balance and membrane integrity by protecting internal water (Liu *et al.*, 2018). The potential involvement of lignin biosynthesis genes in drought tolerance pathways has been proposed for plants (Liu *et al.*, 2018; Xie *et al.*, 2018). Over-expression of cinnamyl alcohol dehydrogenase genes (*CmCAD2* or *CmCAD3*) from melon (*Cucumis melo* L.) in *A. thaliana* was reported to enhance lignin biosynthesis, which was proposed to stabilize internal water content, thereby conferring drought tolerance (Liu *et al.*, 2020). *CmCAD2*- and *CmCAD3*-silenced plants also showed defects in a root Casparian strip and were hypersensitive to drought. In *A. thaliana* it has been shown that the Casparian strip and sclerenchyma cells are mainly composed of lignin or lignin-like polymers and function as an extracellular diffusion barrier of both water and nutrients (Hose *et al.*, 2001;

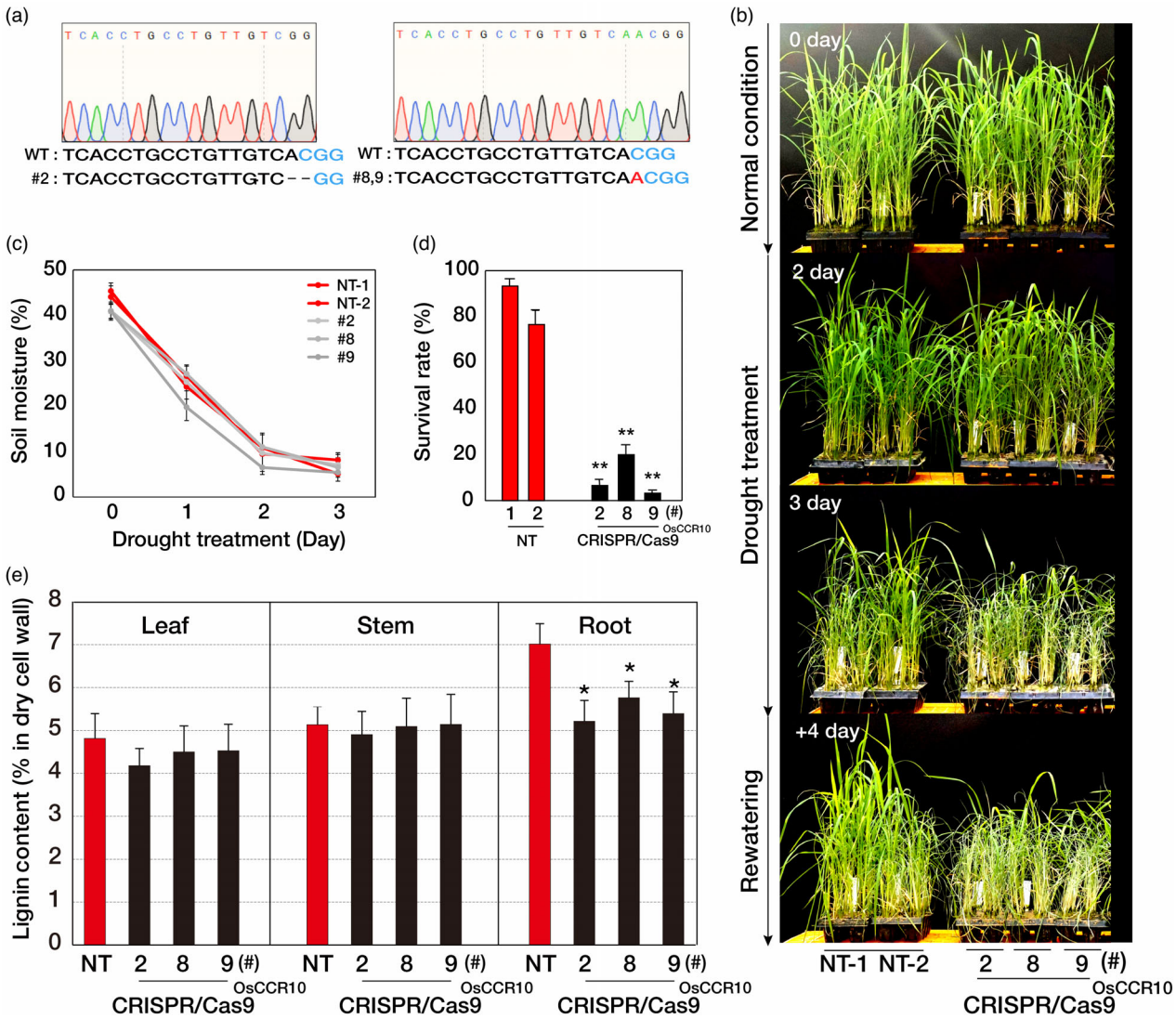

Figure 6 Drought susceptibility of CRISPR/Cas9^{OsCCR10} rice plants. (a) *OsCCR10* mutations in the CRISPR/Cas9^{OsCCR10} transgenic plants (#2, 8 and 9). (b) Phenotypes of CRISPR/Cas9^{OsCCR10} plants under drought stress at the vegetative stage. 5-week-old NT and CRISPR/Cas9^{OsCCR10} mutant plants (#2, 8 and 9) were exposed to drought stress for 3 days, followed by re-watering for 4 days. (c) Soil moisture in the pots exposed to drought treatment at the indicated time points. Values are the means ± SD (n = 10). (d) Survival rate scored 4 days after re-watering. Values are the means ± SD (n = 30). Asterisks indicate significant differences compared with NT (**P < 0.01, one-way ANOVA) (e) Lignin content in 2-month-old CRISPR/Cas9^{OsCCR10} and NT plants. Data bars represent the mean ± SD of three biological replicates (n = 3), each with two technical replicates. Asterisks indicate significant differences compared with NT (P < 0.05, one-way ANOVA).

Naseer *et al.*, 2012). Therefore, lignified cell walls function as waterproof lignin-rich barriers that confer drought tolerance by maintaining the cell osmotic stability and protecting membrane integrity (Liu *et al.*, 2018). In this current study, increases in lignin accumulation were observed in root vascular tissues and sclerenchyma cells in *OsCCR10* over-expressing plants. This root-preferential lignin accumulation likely reduced water loss from the roots to the soil under drought conditions, thereby promoting drought tolerance.

In summary, we showed that *OsCCR10* over-expression in rice plants resulted in increased lignin accumulation in root tissues, leading to enhanced drought tolerance. Furthermore, we showed that *OsNAC5* over-expression promotes the deposition of lignin in roots by directly regulating the expression of *OsCCR10*. Under drought conditions, *OsNAC5* expression is higher and OsNAC5 directly activates the expression of *OsCCR10* in roots. OsCCR10 catalyses the conversion of *p*-coumaroyl-CoA and feruloyl-CoA, precursors of H- lignin and G-lignin, to coumaraldehyde, and coniferaldehyde, respectively, leading to an increase in root lignin content. The resulting accumulation of lignin in roots, especially in the sclerenchyma and fibres, contributes to drought tolerance by reducing water loss (Figure 7).

Experimental procedures

Plasmid construction and *Agrobacterium*-mediated rice transformation

Rice cDNA was derived from RNA isolated from rice (*Oryza sativa* cv. Dongjin) using the Reverse Transcription System

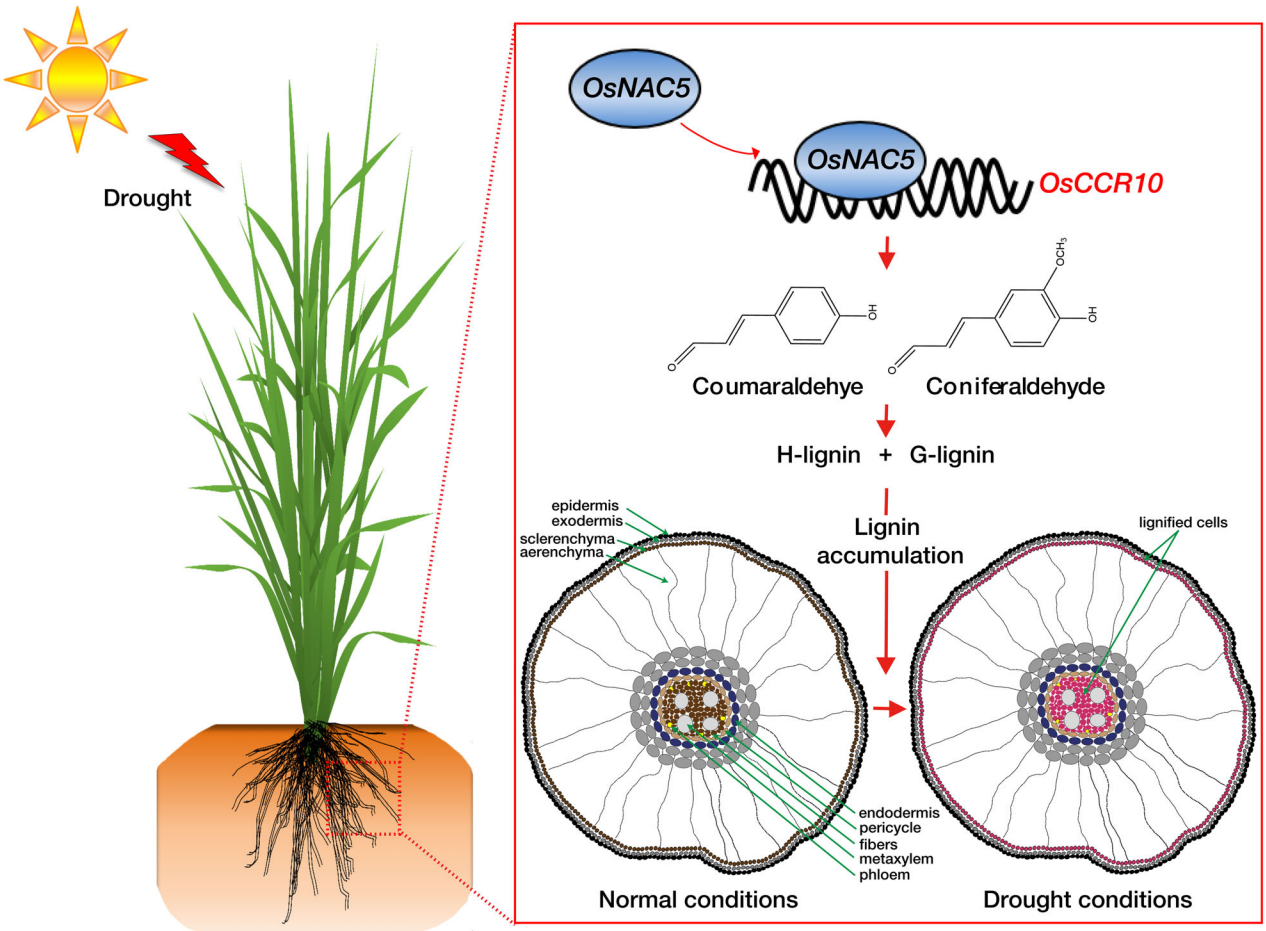

Figure 7 A mechanistic model of the role of *OsCCR10* in drought tolerance. Under drought conditions, *OsNAC5* expression is up-regulated and OsNAC5 activates the expression of *OsCCR10* in roots. OsCCR10 catalyses the reduction of *p*-coumaroyl- CoA and feruloyl-CoA (precursors for H- and G- units of lignin, respectively) to coumaraldehyde and coniferaldehyde, respectively, which results in an increase in root lignin content. The accumulation of lignin, especially sclerenchyma and fibre cells in roots, leads to drought tolerance by reducing water loss.

(Promega, Madison, Wisconsin, USA). The full-length *OsCCR10* cDNA was amplified by PCR with a high-fidelity DNA polymerase PrimeSTAR (TaKaRa, Kyoto, Japan) and used to generate *OsCCR10* (Os02g0811800) over-expression lines. The amplified *OsCCR10* fragment was ligated into the pPZP-GOS2 vector for whole plant expression (*GOS2::OsCCR10*) (Park *et al.*, 2012) and pPZP-RCc3 for root-specific expression (*RCc3::OsCCR10*) (Jeong *et al.*, 2010). For the myc-tagged *OsNAC5* (*PGD1-Myc*^{OsNAC5}) construct, the *OsNAC5* (Os11g0184900) CDS was fused to the 3′ end of a 6xMyc tag sequence in the pE3n vector (Dubin *et al.*, 2008), and the fused fragment was transferred to the *p700PGD1* vector (Park *et al.*, 2012). For the CRISPR/rCas9 (CRISPR/rCas9^{OsCCR10}) construct, a recombinant rice codon-optimized *Streptococcus pyogenes* Cas9 and a single-guide RNA were inserted into the pSB11 vector (Komari *et al.*, 1996) through restriction-enzyme-mediated excision and ligation reactions (Chung *et al.*, 2020). All transgenic rice plants were produced by *Agrobacterium tumefaciens* (LBA4404)-mediated transformation and tissue culturing as previously described (Hiei *et al.*, 1994). Vector maps and primer sequences used in this study are listed in Figure S3 and Table S1, respectively.

Phylogenetic analysis

Phylogenetic analysis was carried out using amino acid sequences from 26 *Oryza sativa* cinnamoyl-CoA reductase proteins using the annotation in the riceXPro public database (http://ricexpro.dna.affrc.go.jp/). Protein sequences from the gene IDs Os02g0808800, Os01g0639200, Os10g0576900, Os01g0283600, Os01g0283700, Os01g0828100, Os06g0623600, Os06g0623300, Os02g0811800, Os02g0812000, Os02g0811400, Os02g0811600, Os09g0491788, Os09g0491820, Os09g0127300, Os08g0277200, Os09g0419200, Os08g0441500, Os02g0180700, Os03g0818200, Os05g0578500, Os09g0262000, Os01g0978400, Os03g0817400, Os06g0623200 and Os08g0183900 were used to construct the phylogenetic tree using the CLC workbench software (https://www.qiagenbioinformatics.com/products/clc-genomics-workbench/).

Subcellular localisation of OsCCR10

For transient expression of OsCCR10-GFP in rice protoplasts, the *OsCCR10* coding region without the stop codon was cloned into the *pHBT* vector (GenBank accession number EF090408) between the 35S promoter and the GFP CDS using the *Bam*HI

and *Not*I restriction enzymes. Primers used for vector construction are listed in Table S1. The vectors introduced into rice protoplasts for transient expression as previously described (Park *et al*., 2015). The *35S::OsCCR10:GFP* plasmid was transformed into the rice protoplasts using the polyethylene glycol-mediated method (Park *et al*., 2015) with ~10^6 cells per reaction. The transformed protoplasts were incubated for 16 h at 28 °C under dark conditions, and the GFP fluorescence of the transfected protoplasts was observed using a confocal laser scanning microscope (Leica TCS SP8 STED, Wetzlar, Germany) as in Park *et al*. (2015).

qRT-PCR analysis

For expression analysis, total RNA was extracted using a Hybrid-R RNA purification kit (GeneAll, Seoul, Korea). cDNA from the total RNA was synthesised using the RevertAid™ First Strand cDNA Synthesis Kit with an oligo(dT) primer (Thermo Fisher Scientific, Waltham, Massachusetts, USA). qRT-PCR was carried out using 2× qRT-PCR Pre-mix with 20× EvaGreen™ (SolGent, Seoul, Korea). The amplification reactions were performed at 95 °C for 10 min, followed by 40 cycles of 95 °C for 20 s, 58 °C for 40 s, 72 °C for 20 s, in a 20 μL mix containing 1 μL of 20× EvaGreen™, 0.25 μM primers and 10 ng cDNA. qRT-PCR analysis was performed using a Stratagene Mx3000p instrument and the Mx3000p software, v2.02 (Stratagene, La Jolla, CA). The analysis was carried out with three biological and three technical replicates. *OsUbi1* (Os06g0681400) was used as an internal control in all experiments. For analysis of *OsCCR10* expression in response to abiotic stresses, rice seeds (*Oryza sativa* cv. Dongjin) were germinated on Murashige and Skoog (MS) medium (Duchefa Biochemie, Haarlem, The Netherlands), transferred to soil and grown for 14 days in a greenhouse at 28 °C. The soil was removed from the seedlings and drought stress was induced by air-drying whole plants. The salinity stress and ABA treatments involved incubating the seedlings in water containing 400 mM NaCl (Sigma-Aldrich, St. Louis, Missouri, USA) or 100 μM ABA (Duchefa), respectively, at 28 °C. Low-temperature stress was induced by placing the seedlings in a 4 °C incubator. The seedlings were harvested at the indicated time points after the abiotic stress treatments. To investigate the spatial and temporal expression patterns of *OsCCR10*, total RNA was extracted from leaves, stems, and roots of 8-, 15-, 30- and 60-day old NT rice plants at the vegetative stages and from leaves and flag leaves at the reproductive stages. Primers used for qRT-PCR are listed Table S1.

Lignin extraction and quantification

Lignin from 2-month-old *GOS2::OsCCR10*, *RCc3::OsCCR10*, and NT leaves, roots and stems was extracted and quantified as previously described (Moreira-Vilar *et al*., 2014). Protein-free cell wall sample (20 mg) was placed into a screw-cap centrifuge tube containing 0.5 mL of 25% acetyl bromide (v/v in glacial acetic acid) and incubated at 70 °C for 30 min. After complete digestion, the sample was quickly cooled in an ice bath, and then mixed with 0.9 mL of 2 M NaOH, 0.1 mL of 5 M hydroxylamine-HCl, and 5 mL of glacial acetic acid sufficient for complete solubilisation of the lignin extract. After centrifugation (1400 g, 5 min), the absorbance of the supernatant was measured at 280 nm. A standard curve was generated with alkali lignin (Aldrich 37, 096-7) and the absorptivity (ε) value obtained was 22.9 g^{-1} L cm^{-1}. The results were expressed as mg lignin per g cell wall.

Phloroglucinol-HCl staining

Hand-cut cross-sections of 2-month-old *GOS2::OsCCR10*, *RCc3::OsCCR10*, and NT stems were stained with phloroglucinol-HCl as previously described (Jensen, 1963). Plant roots were submerged in methanol (>99%) and stored at 4 °C. Root samples were then embedded in a 7% agarose solution and cross-sections were made using a microtome (Leica RM2255) to visualize the root tissues. For the lignin staining, sections were stained with 1% (w/v) phloroglucinol in 95% ethanol for 4 min each.

ChIP-qPCR

ChIP analysis was performed as described in Bowler *et al*. (2004) using 2-week-old rice plants. To isolate protein/DNA complexes, shoots of *PGD1-Myc*^{OsNAC5} and NT plants were cross-linked in a buffer (0.4 M sucrose, 10 mM Tris-HCl [pH 8.0], 5 mM β-mercaptoethanol and 1% formaldehyde) in a vacuum desiccator for 15 min. The cross-linked chromatin was isolated using a sucrose cushion, followed by random shearing for preparation of 100–300 bp of genomic DNA, using 15 cycles of sonication (30 s each). For immunoprecipitation, isolated and sheared chromatin was incubated with polyclonal anti-MYC (sc-789; Santa Cruz Biotechnology, Dallas, TX) and anti-RNA Pol II (sc-33754; Santa Cruz) antisera, while untreated samples were used as controls. Anti-RNA Pol II antibody was used as a transcriptional control. The precipitated protein/DNA complex was collected with protein A agarose (Millipore: Burlington, Massachusetts, USA 16-266), and DNA was purified using the QIAquick PCR purification kit (Qiagen, Hilden, Germany; Chung *et al*., 2018). For ChIP-qPCR, the ChIP product was analysed via quantitative PCR with a Mx3000P RealTime PCR system (Agilent Technologies, Santa Clara, CA). The relative enrichment was normalised with total input. All primer sequences are listed in Table S1.

CCR10 enzymatic activity

OsCCR10 activity was measured according to the methods of Lüderitz and Grisebach (1981). The reaction mixture consisted of 0.1 mM NADPH, 30 μM hydroxycinnamoyl-CoA, and 16, 32 μg of purified recombinant OsCCR10 protein in 100 mM sodium/potassium phosphate buffer (pH 6.25) in a total volume of 500 μL. The enzyme reactions were carried out at 30 °C. The reaction was initiated by addition of recombinant OsCCR10 protein, and A366 of the reaction mixture was monitored for 300 s on a UV/VIS Spectrophotometer V-550 (Jasco, Ishikawa-machi, Tokyo, Japan).

Lignin composition assay

A coil-type CDS Pyroprobe 5000 (CDS Analytical Inc.: Oxford, Pennsylvania,USA) was used for the lignin composition assay of root samples (3-month-old plants). A 1.0 mg sample (extractive free) was introduced to a quartz tube block through quartz cotton on both sides; 2 μL of internal standard (3.9 mg Fluoranthene/1 mL Methanol) was also introduced to the upper quartz cotton for pyrolysis product quantification. The sample was pyrolysed at 600 °C at a heating rate of 10 °C/ms and maintained for 20 s in an inert atmosphere. During pyrolysis, the pyrolyser interface and transfer line were maintained at 250 °C. The pyrolysis products were transferred to a GC/MS (Agilent 7890A/Agilent 5975C, Agilent Technologies Inc., Santa Clara, CA) equipped with a flame ionisation detector (FID) and a DB-5 capillary column (30 m × 0.25 mm × 0.25 μm). The oven was programmed to hold at 50 °C for 5 min, ramp up at 3 °C/

min to 280 °C, and hold for 10 min. The injector and FID temperatures were 250 and 300 °C, respectively. The injector split ratio was set at 15:1. Pyrolysis chemical compounds were qualified using the National Institute of Standard and Technology mass spectra library.

Drought stress treatments and tolerance evaluation

Seeds from *OsCCR10* transgenic and NT control plants (*O. sativa* cv. Dongjin) were sown on MS solid medium and incubated in a dark growth chamber for 4 days at 28 °C. Seedlings were then transferred to a growth chamber with a light/dark cycle of 16 h light/8 h dark and grown for an additional day before transplanting to soil. Thirty plants from each line were transplanted into 10 soil pots (4 × 4 × 6 cm: three plants per pot) within a container (59 × 38.5 × 15 cm) and grown for additional 4 weeks in a greenhouse (16 h light/8 h dark cycle) at 30 °C. Drought stress was imposed by sequentially withholding water. We performed the drought test with separated combinations (NT vs. OE and NT vs. KO) and applied different re-watering time points to observe the difference of drought-tolerant phenotype. *OsCCR10* overexpressing plant (OE) was more tolerant than NT, while *OsCCR10* knockout plant (KO) was more susceptible than NT plants. Therefore, we modulated the re-watering time point differentially. The re-watering time point was 10–12 h earlier in the experiment with NT and KO combination than that in that with NT and OE combination. Drought-induced symptoms were identified by imaging transgenic and NT plants at the indicated time points using a NEX-5N camera (Sony, Tokyo, Japan). The soil moisture contents were measured at the indicated time points using a SM150 Soil Moisture Sensor (Delta-T Devices). Transient chlorophyll a fluorescence was measured using a Handy-PEA fluorimeter (Hansatech Instruments) as previously described (Bang *et al*., 2019). Chlorophyll *a* fluorescence was measured in the longest leaves of each plant after 1 h of dark adaptation to ensure sufficient opening of the reaction centre.

Evaluation of agronomic traits of rice plants grown under field conditions

To evaluate yield components of transgenic and NT plants under field conditions, three independent T_3 homozygous lines of the *GOS2::OsCCR10*, *RCc3::OsCCR10*, and NT control plants were planted in the rice paddy field at the Kyungpook National University, Gunwi (36°06′48.0″N,128°38038.0″E), Korea. Yield parameters were scored from 20 plants collected from two different plots for normal field conditions. To evaluate yield components of the plants under field drought conditions, plants were grown in semi-field conditions under rain-off shelters. Plants were exposed twice to intermittent drought by withholding water during the panicle development stage. After two rounds of drought treatment at the reproductive stage, the plants were re-irrigated until the harvesting stage. Yield components were scored from 20 plants for each line.

Acknowledgments

We thank Prof. Man-Ho Cho of Kyung Hee University for kindly providing us with substrates for CCR10 enzymatic activity experiments. This work was supported by the New Breeding Technologies Development Program (Project No. PJ01477201 to J.-K.K.) and Cooperative Research Program for Agriculture Science and Technology Development (Project No. PJ01566801 to J.S.S), Rural Development Administration, Republic of Korea.

Conflict of interest

The authors declare no conflict of interest.

Author contributions

S.W.B., S.C., and J.-K.K. designed the experiments, and S.W.B., S.C., S.E.J., and J.S.S. performed the experiments. X.J. and J.W.C. analyse the lignin composition in transgenic plants. S.W.B., J.S.S. and J.-K.K. wrote the manuscript and prepared the figures.

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Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Figure S1 Expression patterns of rice *OsCCRs* in response to drought stress.
Figure S2 Protein levels in *PGD1:OsNAC4-MYC* transgenic plants.
Figure S3 Vectors used in this study.
Figure S4 Lignin contents of NT plants (Dongjin) under drought conditions.
Table S1 Primers used in this study.
Table S2 *GOS2::OsCCR10* and *RCc3::OsCCR10* agronomic traits transgenic rice plants under normal and drought conditions.

