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Characterization of the *OsCCR10*, a Lignin Biosynthesis Gene Involved in Rice Drought Tolerance Mechanism

가뭄 저항성 메커니즘에 연관된 리그닌 생합성 유전자,

*OsCCR10*의 규명

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Seowon Choi

Department of International Agriculture Technology

Graduate School of International Agriculture Technology

Seoul National University

Abstract

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Seowon Choi

Major of International Agricultural Technology
Department of International Agricultural Technology
Graduate School of International Agricultural Technology
Seoul National University

Drought is a major destructive environmental stress causing remarkable reductions in crop yield worldwide. Plant have developed morphological, physiological and biochemical mechanisms that enable them to cope with negative effects of drought stress. Lignin is a major constituent of the cell walls and modification of cell wall structure by accumulating lignin is required for plants to adjust drought stress. Our previous study demonstrated that *OsCCR10 (Oryza sativa CINNAMOYL-COA REDUCTASE 10*) is a strong candidate which causes drought-induced lignin accumulation. In this study, I characterized molecular functions of *OsCCR10* in lignin-mediated drought tolerance mechanisms. *OsCCR10* expression was induced in roots of rice plants by drought, ABA and high salt treatments. Overexpression of *CCR10* either whole body and root specific significantly increased drought tolerance in rice plants. OsCCR10 was detected in cytoplasm in rice protoplasts where lignin biosynthesis occurs. Enzyme assay revealed that OsCCR10 catalyzed reductions of coumaroyl-CoA and feruloyl-CoA to coumaraldehyde and

coniferaldehyde. In *OsCCR10* overexpressing plants, strong staining of lignin was found in outer cell layer including epidermis and vascular tissues. Moreover, lignin quantification showed that overexpression of *OsCCR10* was sufficient to increase total lignin content in leaves, stems and roots of transgenic plants. Taken together, I suggest that *OsCCR10* mediates drought-induced accumulation of lignin and overexpression of *OsCCR10* confers drought tolerance in rice. This study will provide a leading candidate to overcome drought stress by controlling lignin biosynthesis.

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Keyword: Rice, drought tolerance, *OsCCR*, lignin biosynthesis, cell wall

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Introduction

The World's food demand is a critical issue in these days. Rice (*Oryza sativa japonica*) is one of the major and staple crop in the world. Since world population is increasing fast, global rice yields must be increased as growing population and food demands. Food problem is not only initiated from increasing world population but also challenging from climate changes which occurring worldwide (Boyer et al., 1982). Abiotic stresses, including drought, cold and salinity are the major limitation for crop production and can reduce crop yields (Boyer et al., 1982; Oreke et al., 2006). To deal with abiotic stresses, plants have developed a wide variety of defense mechanisms such as stiff the cell walls, as well as activation defense-responsive genes (SOD, POD, CAT, APX and GR enzymes) and hormonal regulation.

Drought (water stress) is one of the most important environmental stresses, caused wide range changes in the physiological morphological, biochemical and molecular changes in plants (Pandey et al., 2015). Therefore, there are considerable interests in developing drought tolerant crops which can grow in harsh environments. Drought stress is caused by imbalance between water uptake and evaporation (Moura et al., 2010). One of the plant avoidant strategies is depositing hydrophobic lignin in their cell walls when exposed to drought stress (Yoshimura et al., 2008). Plant cell wall is the first barrier against stresses and its modification can modulate plant growth and change components of cell wall with an impermeable physical barrier (Miedes et al., 2014). However, the molecular mechanisms behind the response to drought stresses are not fully understood. One of the general reactions of plants from biotic and abiotic stresses is lignin biosynthesis. (Moura et al., 2010; Liu et al., 2015).

One of the plant's organic polymers, lignin is important for structural support of the cell wall and strengthen the plant stiffness. Lignin is one of the most important secondary metabolite, which is created by the phenylalanine mediated lignin biosynthesis pathway in plant cells and makes cell wall stiffen and strength (Ralph et al., 2004). Many studies suggest that abiotic stress tolerance is related with plant lignification. In rice, overexpression of *OsTF1L*, a rice homeodomain-leucine zipper transcription factor gene, promotes lignin biosynthesis and it improves drought tolerance (Bang et al., 2018). In maize, drought-tolerant inborn plants with high expression levels of lignin biosynthesis genes (Hu et al., 2009). Therefore, high levels of lignification can enhance drought tolerance. Lignin biosynthesis is a very complicated mechanism that is separated into three processes. ⅰ) biosynthesis of lignin monomers, ⅱ) transport, ⅲ) polymerization as presented in Fig. 1. Lignin monomers are synthesized in cytoplasm and transported to apoplast and then polymerized in the cell wall (Miao et al., 2010).

Lignin was composed by complex aromatic polymers, *p*-hydroxyphenyl (H), guiacyl (G) and sinapyl (s) units which are derived from monolignols in secondary cell wall (Alejandro et al., 2012; Bonawitz et al., 2010; Liu et al., 2011; Miao et al., 2010). Their deposition occurs in the cell walls and allow moving water and nutrient as well as mechanical structural support. Lignin can control water penetration and transpiration through plant cell wall, which helps to maintain cell osmotic balance (Monties et al., 2005). And lignin helps cell wall waterproof, makes it possible to transport of water through the vascular system (Schuetz et al., 2014). Increase in lignification is a common stress response connected with biotic and abiotic stresses (Moura et al., 2010). The amount and composition of lignin vary cell types and individual cell wall layers are influenced by developmental and environmental situations. Deposition of lignin occurs in the walls of certain tissue providing additional strength and water impermeability. The biosynthesis of lignins begins with phenylpropanoid pathway starting with phenylalanine and catalyzing to

hydroxycinnamoyl-CoA esters which are common precursors of monolignol before forming lignins (Hahlbrock and scheel et al., 1989; Dixon and Lamb et al., 1990).

Cinnamoyl CoA Reductase (CCR) is the first enzyme catalyzing the initial step in monolignol biosynthesis (Lauvergeat et al., 2001), and plays a key role in lignin formation, leads to a vital role in plants both for structural development and defense response (Barros et al., 2015). Cinnamoyl-CoA esters are catalyzed in the lignin biosynthesis pathway by cinnamoyl-CoA enzyme to produce monolignols through reductive steps, as briefly presented in figure 1 (Lauvergeat et al., 2001). In the monolignol pathway, catalyzing the modification of coumaroyl-, feruloyl-, and sinapoyl-CoAs to coumaraldehyde, coniferaldehyde, and sinapaldehyde, respectively (Gross et al., 1981; Davin et al., 2008; Vanholme et al., 2010). Lignin biosynthesis during plant development, plant lignification also increases as the expression of CCR enzyme increased in various tissues (Lancombe et al., 1997, 2001). Additionally, *CCR* is induced under conditions such as wound or pathogen infection (Lauvergeat et al., 2001; Kawasaki et al., 2006). Homologs of *CCR* genes have been reported in various plants. In case of maize, two *CCR* enzymes, *ZmCCR1* and *ZmCCR2*, have been reported (Pichon et al., 1998). Although Zm*CCR*1 was expressed in lignification of tissue, Zm*CCR*2 was found mainly in roots and it was induced by drought conditions (Fan et al., 2006). There are 11 genes in *Arabidopsis thaliana*, nine in *Popilis trichocarpa*, 26 in Rice (*Oryza sativa*) and 10 in *Eucalyptus* (Costa et al., 2003; Kawasaki et al., 2006; Shi et al., 2010; Carocha et al., 2015). In maize (*Zea mays*), drought-tolerant lines with high expression level of lignin biosynthesis genes like *cinnamyl alcohol dehydrogenase* (*CAD*), caffeate O-methyltransferase (COMT), which are working as mediator in lignin biosynthesis pathway, show high lignification in shoots. And they are suggesting that a relationship between drought tolerance and lignification. The previous study reported that, watermelon (*Citrullus lanatus*) increases drought tolerance by upregulating the expression of lignin biosynthetic

genes after exposed to drought stress (Yoshimura et al., 2008). An important lignin biosynthesis gene *CCR* coordinated with lignin accumulation in developing plant.

In this study, we generated overexpression transgenic lines of OsCCR10, a lignin biosynthesis gene, and found that the overexpression lines enhanced drought tolerance, while non-transgenic lines remain sensitive to drought stress. Overexpressing of *OsCCR10* increases accumulation of lignin in plants. This *OsCCR10*-mediated drought tolerance mechanism increases co-related information between lignification and drought tolerance in rice.

Materials and Methods

1. Plasmid construction and rice transformation

For the overexpression of genes in rice, the full-length cDNA of *CCR10* (Os02g0811800) was amplified by PCR according to the manufacturer's instructions (Promega, Madison,WI). The construct which was sub-cloned into the p700 rice transformation vector carrying *GOS2* (whole body overexpression) and *RCc3* (root specific overexpression) were named *GOS2::OsCCR10* and *RCc3::OsCCR10*. For transient expression of *OsCCR10-GFP* in rice protoplasts, the *OsCCR10* coding sequence without the stop codon was inserted into the p700 rice transformation vector (Lee et al., 2015) between the *GOS2* promoter (De Pater et al., 1992) and the *GFP* coding sequence. Transgene expression levels in T_0 transgenic lines were determined using the qRT-PCR and selected the best five overexpressing lines for *GOS2::OsCCR10* and *RCc3::OsCCR10*. At T_1 generation, homozygous lines were selected through growth in phosphinothricin-containing MS media. Transgenic lines with somaclonal variations were eliminated by successive field selection through the T_3 generation. Finally, we obtained three independent homozygous lines of *OsCCR10* overexpression plants (#8, 9 and 13 for whole expression promoter; #2, 16 and 27 for root specific expression promoter).

2. Preparation of Phylogenetic Tree

Analyze protein sequences of functional CCRs identified from other plant species were searched from Rice X Pro (http://ricexpro.dna.affrc.go.jp) and the National Center for Biotechnological Information (https://www.ncbi.nlm.nih.gov) database,

respectively. Multiple amino acid sequence alignment was performed with CLC sequence viewer using the neighbor-joining method.

3. RNA extraction and cDNA synthesis

Total RNA was extracted using a Hybrid-R RNA purification kit (GeneAll). 1 µg of total RNA was used to synthesize cDNA in a 20 µL reaction using the Superscript II cDNA synthesis system (Invitrogen). As RNA is unstable, cDNA synthesis is recommended for better RT-PCR result. Before cDNA synthesis, the concentration of each RNA should be set into same concentration using NANO DROP 2000c spectrophotometer (Thermo scientific). The 1 µg of RNA was diluted in 11.5 µL of water. Oligo primer (Oligo dT) 1 µL is added and incubated at 70°C for 5 min, then chilled on ice (4°C). The master mix should be made in order as 5x reaction buffer 4 µL, RiboLock™ Ribonuclease Inhibitor (20 ug/µL) 0.5 µL, 10 mM dNTP mix 2 µL, Reverse transcriptase (20 ug/µL) 2 µL for the final volume 20 µL. The mixture is incubated at 42°C for 90 min and the reaction is stopped by heating at 70°C for 10 min and then chill on ice. cDNA is diluted by adding 80 µl of dH_2O.

4. qRT- PCR analysis and plants preparation for abiotic stress assay

For gene expression level check, qRT-PCR was carried out using 2 x qRT-PCR Pre-mix with 20 x EvaGreen™ (SolGent, Seoul, Korea). The amplification reactions were performed at 95°C for 10 min, followed by 42 cycles of 95°C for 15 s, 60°C for 60 s, 72°C for 20 s, in a 20 µL mix containing 1 µL of 20 x EvaGreen™, 0.25 µM primers and 10 ng cDNA. qRT-PCR analysis was performed using a Stratagene Mx3000p instrument and Mx3000p software, v2.02 (Stratagene, La Jolla, CA). The rice *ubiquitin1* transcript was used as a normalization control, and two technical and three biological replicates were analyzed for all quantitative experiments. For

analysis of *CCR10* expression patterns in response to various abiotic stresses, NT plants (*Oryza sativa* L. var. Japonica cv. Dongjin) were first grown in soil for 2 weeks under greenhouse conditions (16 h light/8 h dark at 28-30ºC). The drought treatment consisted of air-drying whole plants by removing soil for indicated time points. For the ABA, NaCl and low temperature treatments, whole plants were transferred to water containing 100 μM ABA or 400-mM NaCl, or were exposed to 4°C for the indicated time points.

To investigate the spatial and temporal expression patterns of *OsCCR10*, total RNA was extracted from shoots and roots of 2-week-old NT rice plants at the vegetative stages. To measure *OsCCR10* expression levels in *OsCCR10*-overexpressing total RNA samples were extracted from the shoots of 2-week-old transgenic and NT rice seedlings.

5. Drought tolerance evaluation at vegetative stage

OsCCR10 transgenic and NT rice plants were germinated on Murashige-Skoog (MS) media in a growth chamber in the dark at 28°C for 3 days, followed by light conditions at 30°C for 2 days. Thirty plants of each transgenic line and NT control plants were transplanted to soil pots (4 x 4 x 6 cm; 3 plants per pot) and grown for 6 weeks in a glasshouse (16 h light/8 h dark) at 28–30°C. Pots were moved from the container for 3days drought treatment and returned into the container for re-watering until plant showing recovery from drought stress. Soil moisture was measured throughout the experiment to confirm similar water-deficit conditions using a soil moisture sensor (SM150, Delta-T Devices) for 3 days. To measure of Chlorophyll Fluorescence, same 6-week-old plants used for drought treatment test. At the indicated time point, after three-hour dark-adaptation, the tallest and the healthy-looking leaves from each plant were selected and measured at their apex, middle and base parts using the Handy-PEA fluorimeter (Hanstech Instrument, UK). Twenty-five readings per

one line were averaged using the Handy PEA software (version 1.31). Chlorophyll a fluorescence (F_v/F_m) was measured and analyzed according to the equations of the Jip test. Drought-induced symptoms were visualized by taking pictures at the indicated time points using a NEX-5N camera (Sony, Japan). Soil moisture was measured using SM150 (Delta T Devices, UK) at the indicated time points.

6. Vector construction and transformation of rice protoplasts

The *OsCCR10* was amplified by PCR using a high-fidelity DNA polymerase PrimeSTAR (TaKaRa). The full-length cDNAs of *OsCCR10* was amplified from cDNA by PCR. The primers used for cloning were forward 5′- TTG CTC CGT GGA TCC ATG TCG TCC AAC AAT TCC AT-3′ and reverse 5′- AAA GCG GCC GCA AAT CAA ACA TGC ACG CTG TTG TGC CAC-3′ for *OsCCR10.* For transient expression of *OsCCR10*, *CCR10* coding region without stop codon was cloned into pHBT vector between 35S promoter and GFP coding sequence using the *BamH1* and *Not1* restriction sites. Protoplast isolation from shoots of 11- day- old rice seedlings (*O. sativa* cv. Ilmi). The construct, *35S::CCR10-GFP* was transformed into isolated rice protoplasts using PEG (polyethylene glycol)-mediated transformation. Isolation of protoplasts and PEG-mediated transformation were performed as described. (Jung et al., 2015)

7. Subcellular localization of *OsCCR10* using rice protoplasts

To check subcellular expression experiments, plasmids containing the *OsCCR10* coding sequence fused to the GFP reporter coding sequence were transformed into isolated rice protoplasts using PEG-mediated transformation (Jung et al., 2015). GFP and mCherry signals were detected using a Leica SP8 STED laser scanning confocal

microscope (Leica, Solms, Germany). Peroxisome, golgi and endoplasmic reticulum specific genes fused with mCherry were used (Brook K. Nelson et al., 2007).

8. Phenotypic analysis and 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). Plants roots were submerged in methanol (>99%) and stored at 4°C. Embedding root samples in a 7% solution of agarose. To visualize the root tissues, cross sections were made using rotary microtome (Leica RM2255). To stain lignin specifically, sections were stained with 1% (w/v) phloroglucinol in 95% ethanol for 4 mins each.

9. Lignin extraction and quantification

Lignin was extracted and quantified as previously described (Moreira-Vilar et al.,2014), with minor modifications. For protein-free cell wall preparations, leaves, roots and stems of two-month-old *GOS2::OsCCR10*, *RCc3::OsCCR10* and NT were dried in an oven at 80°C until a constant weight was achieved. Dry samples (0.3 g) were homogenized with 7 mL of 50 mM potassium phosphate buffer (pH 7.0) and the pellet was centrifuged and washed as follows: two times with 7 mL of phosphate buffer (pH 7.0), three times with 7 mL of 1% (v/v) Triton X-100 in phosphate buffer (pH 7.0), two times with 7 mL of 1 M NaCl in phosphate buffer (pH 7.0), two times with 7 mL of distilled water, two times with 5 mL of acetone. The final pellet was dried in an oven at 60°C for 24 h and then in a vacuum desiccator. The pellet (20 mg) were mixed with 500 µL of 25% acetyl bromide (1:1 v/v in glacial acetic acid) and incubated at 70°C for 30 min. The samples were then immediately cooled on ice and 900 µL of 2 M NaOH with 100 µL of 5 M hydroxylamine-HCl was added to each.

Thereafter, 4 mL of glacial acetic acid was added to each to solubilize the lignin. The samples were then centrifuged and the absorbance of the supernatant was measured at 280 nm. The lignin concentration was estimated using a standard curve generated with alkali lignin (Sigma-Aldrich, 370959). The values were expressed as mg of lignin per gram of cell wall (with the latter corresponding to the mass of the original pellet).

10. Cloning of OsCCR10 for recombinant protein

Total RNA was isolated from 8-week-old rice leaves using Hibrid-R (GeneAll, Korea). The first cDNA was synthesized from 1ug of the total RNA using RevertAid M-MuLV Reverse Transcriptase (Thermo Scientific, USA). Cloning primers for *OsCCR10* gene were designed according to the sequences in the MSU RGAP database. The primers which were used for cloning are provided in table 2. PCR was performed using PrimeSTAR® HS DNA polymerase (Takara, Japan). Using the In-fusion system (Takara, Japan), PCR product was cloned into linearized pET28a(+) vector and construct was transformed into E.coli TOP10 cells. And after sequence check, the *OsCCR10*/pET28a(+) construct was transformed into E. coli BL21(DE3) cells for heterologous expression of *OsCCR10*.

11. Expression and Purification of Recombinant OsCCR10

The E. coli transformants harboring the OsCCR10/pET28a(+) construct were grown at 37°C until an OD600 of ~0.6 in LB medium containing kanamycin (25 μg/mL) was achieved. At that point, 0.1 mM isopropyl b-D-thiogalactopyranoside (IPTG) was added in the culture for induction. After additional incubation at 20°C for 16 h, the cells were harvested by centrifugation (5,000 g for 15 min). Cell pellets were resuspended in phosphate-buffered saline (137 mM NaCl, 2.7 mM KCl, 10mM Na_2HPO_4, 2 mM KH_2PO_4) supplemented with lysozyme (1 mg/mL) and phenylmethylsulfonyl

fluoride (1mM). The resuspended cells were sonicated on ice, and the crude protein extracts were obtained by centrifugation (15,900 g for 20 min, 4°C). The crude protein samples were mixed with Ni-NTA Agarose beads (Qiagen, Hilden, Germany) and incubated at 4°C for 2 h with agitation. The mixtures were packed into a chromatography column and washed three times with a five-column volume of 20 mM imidazole in Tris buffer (5 0mM Tris, pH 8.0, 300 mM NaCl). The recombinant OsCCR10 was eluted with 40–250 mM imidazole in Tris buffer. The eluted proteins were analyzed by sodiumdodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

12. CCR10 Enzymatic Activity Assay

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) to a total volume of 500 μL. The enzyme reactions were carried out at 30°C. The reaction was initiated by an addition of recombinant OsCCR10 protein, and A366 were monitored for 300 sec by a UV/VIS Spectrophotometer V-550 (Jasco, Ishikawa-machi, TOKYO, Japan).

Results

1. *OsCCR10* is involved in *OsERF71*-mediated lignification in Rice

Previously, we investigated expression profile of *OsERF71* overexpressing transgenic plants (Lee DK et al., 2016). *OsERF71* gene, a AP/ERF transcription factor, mediates drought tolerance by regulating expression of genes involved in cell loosening and lignin biosynthesis in rice. It was found that *OsCCR10* is involved in *OsERF71*-mediated lignin accumulation (Fig. 2A). To corroborate the relationship between *OsERF71* and *OsCCR10*, I re-analyzed the expression patterns of *OsCCR10* in *OsERF71* overexpressing transgenic plants. *OsCCR10* expression was up-regulated in both *GOS2::OsERF71* and *RCc3::OsERF71* overexpressing transgenic plants. In addition, the expression level was down-regulated in RNAi-mediated *OsERF71* suppressing plants (*GOS2::OsERF71*^{RNAi}line) (Fig. 2B). These data indicate that the transcription factor *OsERF71* modulates down-stream genes, including *OsCCR10* for accumulation of lignin, that may contribute to improve drought stress tolerance. Based on these results, I hypothesized that increased lignification confers drought tolerance in rice. To confirm this hypothesis, here I characterized function of *OsCCR10* (a lignin biosynthetic enzyme) in lignin biosynthesis and drought tolerance in rice.

2. Phylogenetic analysis of *OsCCR10*

To characterize functional domains of the CCR10 protein, we analyzed full-length amino acid sequences of all rice CCR proteins. *OsCCR* is divided into two major groups. NAD-dependent and dehydrogenase groups as presented in table 1. In N-terminal domain, a parallel beta sheet structure serves as a binding domain for NADPH (Pan et al., 2014), while the second domain which is in C-terminal, it is

consisted of several alpha helices, beta strands, and extended loops, is responsible for binding the cinnamoyl-CoA substrate (Sattler et al., 2017). The *OsCCR10* belongs to the dehydrogenase group. Additionally, phylogenetic tree of OsCCR10 and other CCR proteins from rice and other species of plant revealed that *OsCCR10* forms a subgroup with *OsCCR1* (Fig. 3)

3. *OsCCR10* is induced by drought in roots

To investigate functions of *OsCCR10* in abiotic stress response, expression patterns of *OsCCR10* was examined in rice plants treated with various abiotic stresses, such as drought, ABA, high salinity and low temperature (Fig. 4A). The results showed that *OsCCR10* expression was mainly induced in roots of rice plant after drought, salt and ABA treatments. However, in case of low temperature, *OsCCR10* expression was only induced in leaves in rice plants. Next, tissue specific expression patterns of *OsCCR10* was analyzed by using various tissues of rice plants at different developmental stages (Fig. 4B). It was found that *OsCCR10* showed root-specific expression patterns and the highest expression level was detected at reproductive stage of the growth. Even though the expression level of *OsCCR10* in shoots was lower than roots, it was confirmed that the basal level of *OsCCR10* was expressed. These results showed that *OsCCR10* is mainly expressed in roots and its expression was induced by drought stress.

4. Subcellular localization of *OsCCR10*

To determine subcellular localization of OsCCR10, a construct expressing the coding sequence of *OsCCR10* translationally fused with green fluorescent protein (GFP) under the control of *35S* promoter (*35S::OsCCR10-GFP*) was generated (Fig. 5A). The markers for peroxisome, Golgi and endoplasmic reticulum were used to

visualize subcellular organelles (Brook et al., 2007). GFP fluorescence was broadly observed in protoplasts and cytoplasmic membrane, and no obvious overlap was detected with tested subcellular organelle markers (Fig. 5B). It indicates that OsCCR10 is cytosolic protein.

5. Generation of *OsCCR10* overexpressing transgenic rice plants

For characterizing the function of *OsCCR10*, the overexpression vector was generated by fusing the cDNA of *OsCCR10* with the *GOS2* (*GOS2::OsCCR10*) for whole-body overexpression of *OsCCR10* and *RCc3* promoter (*RCc3::OsCCR10*) for root-specific overexpression of *OsCCR10* (Fig. 6A). qRT-PCR was performed to check *OsCCR10* expression levels in roots of *GOS2::OsCCR10* and *RCc3::OsCCR10* transgenic rice plants (Fig. 6B). Based on *OsCCR10* expression level, three independent line of *GOS2::OsCCR10* and *RCc3::OsCCR10* were selected and propagated in fields to obtain T_3 generation. Expression levels of *OsCCR10* was confirmed in selected T_3 homozygous lines of *GOS2::OsCCR10* and *RCc3::OsCCR10* transgenic plants (Fig. 6C). Increased expression levels of *OsCCR10* gene were detected in both leaves and roots of *GOS2::OsCCR10* transgenic plants compared to NT plants. In *RCc3::OsCCR10* transgenic plants, overexpression of OsCCR10 was detected in roots but not shoots, confirming root-specific overexpression of *OsCCR10* in *RCc3::OsCCR10* transgenic plants. In case of T_3 generation plants, *GOS2::OsCCR10* and *RCc3::OsCCR10* expression levels in shoot were 40 to 680 times higher than NT control plants. And *GOS2::OsCCR10* expression levels in root were similar level to NT control plants. But in case of *RCc3::OsCCR10*, the expression levels were 15 to 95 times higher than NT control plants (Fig. 6C).

6. Overexpression of *OsCCR10* improves drought tolerance in transgenic rice plants

To evaluate the tolerance of *OsCCR10* overexpressing transgenic rice plants in drought conditions, 6-week-old non-transgenic (NT), *GOS2::OsCCR10* and *RCc3::OsCCR10* transgenic plants were grown in greenhouse and subjected to drought stress by withholding water for 3days, and followed by re-watering for 7 days. As the time course of drought treatments, drought induced symptoms, such as leaf rolling, wilting, and loss of chlorophyll appeared earlier in NT plants compared to those in *GOS2::OsCCR10* and *RCc3::OsCCR10* transgenic plants (Fig. 7A). Soil moisture content was similarly decreased and reached about 10% after 3 days of drought treatments, indicating that stress treatments were uniformly applied across the plants (Fig. 7B). After re-watering, *GOS2::OsCCR10* and *RCc3::OsCCR10* transgenic plants were rapidly recovered from drought induced symptoms, while NT plants continuously withered (Fig. 7A). *GOS3::OsCCR10* and *RCc3::OsCCR10* transgenic plants showed 80 to 100% survival rate at 7 days after re-watering whereas NT plants only showed 10% (Fig. 7C). Since drought stress negatively affects photosynthetic efficiency of plants, the degree of drought tolerance was further analyzed by measuring photochemical efficiency (F_v/F_m) in plants. F_v/F_m values of NT plants from 3 days after drought treatments were rapidly decreased while those of *GOS2::OsCCR10* and *RCc3::OsCCR10* plants slightly decreased (Fig. 7D). Based on these results, overexpression of *OsCCR10* enhances drought tolerance in rice plants.

7. Cloning and expression of OsCCR10 protein

To explain biochemical functions of OsCCR, we attempted to express recombinant OsCCR10 protein which can be used for enzyme assay. The coding sequence of *OsCCR10* amplified from rice cDNA was cloned into linearized pET28a(+) vector (Fig. 8A). OcCCR10 recombinant protein was successfully

expressed as soluble protein in *E. coli* by 0.1 mM IPTG at an induction temperature of 22°C. Recombinant OsCCR10 were purified with Ni^{2+} affinity chromatography, and the purified OsCCR10 protein was analyzed by SDS-PAGE. OsCCR10 recombinant protein exhibited molecular mass of 37.6 kDa on SDS-PAGE, which was in agreement with its theoretical molecular mass (Fig. 8B).

8. Enzyme activity of OsCCR10

To examine the enzymatic property of OsCCR10, the activity of recombinant OsCCR10 was assayed using hydroxycinnamoyl-CoA substrates, which are consist with coumaroyl-, feruloyl-, and sinapoyl-CoA, precursors for the H-, G-, and S units of lignin (Fig. 9A). The Y-axis of the graph indicates absorbance of the NADPH measured by UV-spectrometer. Since NADPH is a cofactor of reduction of hydroxycinnamoyl-CoA by CCR, the decrease of NADPH indicates measurement of enzymatic activity of CCR. The X-axis is the time for enzyme activity reaction. The enzyme assay revealed a greater catalytic activity of *OsCCR10* toward coumaroyl- and feruloyl-CoAs than sinapoyl-CoA. Decreasing graphs of coumaroyl- and feruloyl-CoA show that *OsCCR10* works as a reductase. The substrates decreased by about 12% by adding 30mM of coumaroyl-CoA and 16 μg of enzyme, and the substrates decreased by about 28% when 32 μg of enzyme was added. In case of feruloyl-CoA, the substrates decreased by about 3% when the substrates were added 30mM and the enzyme 16 μg, and when 32 μg of enzyme , the substrates decreased by about 7.7%. In the case of sinapoyl-CoA, the graph does not decrease, so it can be determined that *OsCCR10* enzyme has no activity to sinapoyl-CoA. This result indicates that among three hydroxycinnamoyl-CoA substrates, *OsCCR10* has substrate preferences for coumaroyl- and feruloyl-CoAs. As the experiment was conducted by adding enzyme twofold, the results were same. This result indicates that

among three hydroxycinnamoyl-CoA substrates, OsCCR10 has substrate preferences for coumaroyl-CoA and feruloyl-CoA (Fig. 9B and Table 3).

9. OsCCR10 overexpression significantly increases lignin accumulation

To investigate effects of *OsCCR10* overexpression in lignin accumulation in plants, phloroglucinol-HCl staining was performed with NT, *GOS2::OsCCR10* and *RCc3::OsCCR10* transgenic plants to visualize accumulation of lignin in plants. Since expression of *OsCCR10* was higher in 2-month-old rice plant (Fig. 4B), I used 2-month-old plants for checking differences of lignin accumulation between NT and the transgenic plants. Strong red staining pattern corresponding accumulation of lignification was found in roots of both *GOS2::OsCCR10* and *RCc3::OsCCR10* transgenic plants compared with NT control plant (Fig. 10A). In particular, the metaxylem, endodermis, sclerenchyma and epidermis of the root of the *GOS2::OsCCR10* and *RCc3::OsCCR10* transgenic plants were highly lignified, while the same tissues of the NT plants remained less lignified. In the case of stem, the epidermis and endodermis of the stem, as well as the vasculature bundles and sclerenchyma tissues and of the endodermis of the *GOS2::OsCCR10* transgenic plants were highly lignified, whereas the corresponding tissues of the *RCc3::OsCCR10* transgenic and NT plants remained less lignified. (Fig. 10B, C). To confirm this staining data, I quantified lignin contents in NT, *GOS2::OsCCR10* and *RCc3::OsCCR10* transgenic plants using acetyl bromide method (Moreira-Vilar et al., 2014). Lignin amounts were higher in roots of the *GOS2::OsCCR10* and *RCc3::OsCCR10* transgenic plants than NT control plants (Fig. 11, Table. 4). *GOS2::OsCCR10* transgenic plants also showed higher accumulation of lignin in leaves and stems. In contrast, lignin levels in stems and leaves of *RCc3::OsCCR10* transgenic plants remained similar level with NT plants. Taken together, our results

indicate that *OsCCR10* overexpression is sufficient to increase lignification through lignin biosynthesis in rice.

Table 1. Lists of annotated *CCR*s in rice plants

Name	Locus ID	Gene description	CDS length (nucleotides)	Protein length (amino acids)
OsCCR1	Os02g56460	Cinnamoyl-CoA reductase, dehydrogenase, putative, expressed	1,017	338
OsCCR2	Os01g45200	NAD-dependent epimerase/dehydratase, putative, expressed	1,092	363
OsCCR3	Os10g42620	NAD-dependent epimerase/dehydratase, putative, expressed	960	319
OsCCR4	Os01g18110	Cinnamoyl-CoA reductase, putative, expressed	981	326
OsCCR5	Os01g18120	cinnamoyl CoA reductase, putative, expressed	987	328
OsCCR6	Os01g61230	NAD-dependent epimerase/dehydratase, putative, expressed	981	326
OsCCR7	Os06g41840	Cinnamoyl-CoA reductase, dehydrogenase, putative, expressed	966	321
OsCCR8	Os06g41810	NAD-dependent epimerase/dehydratase, putative, expressed	966	321
OsCCR10	Os02g56700	Cinnamoyl-CoA reductase, dehydrogenase, putative, expressed	1,020	339
OsCCR11	Os02g56720	NAD-dependent epimerase/dehydratase, putative, expressed	1,005	334
OsCCR12	Os02g56680	NAD-dependent epimerase/dehydratase, putative, expressed	1,014	337
OsCCR17	Os09g04050	Cinnamoyl-CoA reductase, dehydrogenase, putative, expressed	1,044	347
OsCCR18	Os08g17500	Cinnamoyl-CoA reductase, dehydrogenase, putative, expressed	1,029	342
OsCCR19	Os09g25150	NAD-dependent epimerase/dehydratase, putative, expressed	1,074	357
OsCCR20	Os08g34280	Cinnamoyl-CoA reductase, dehydrogenase, putative, expressed	1,086	361
OsCCR21	Os02g08420	cinnamoyl CoA reductase, putative, expressed	1,035	344
OsCCR22	Os03g60380	NAD-dependent epimerase/dehydratase, putative, expressed	1,005	334
OsCCR23	Os05g50250	NAD-dependent epimerase/dehydratase, putative, expressed	1,140	379
OsCCR24	Os09g08720	Cinnamoyl-CoA reductase, putative, expressed	975	324
OsCCR26	Os01g74660	NAD-dependent epimerase/dehydratase, putative, expressed	984	327
OsCCR28	Os06g41800	Cinnamoyl-CoA reductase, , putative, expressed	435	144
OsCCR29	Os08g08500	NAD-dependent epimerase/dehydratase, putative, expressed	690	229

Table 2. List of primer sequences used in this study

PCR Primer	5' to 3'	Strand
qRT-PCR		
OsCCR10	ATGGCGATGATGAGAAGAAGCA	Forward
OsCCR10	CACAGTGTCAGCCTCTCCTTG	Reverse
Subcellular localization		
K18_Inf2_F_BamH I	TTGCTCCGTGGATCCATGTCGTCCAACAATTCCAT	Forward
K18_Inf2_R_Not I	AAAGCGGCCGCAAATCAAACATGCACGCTGTTGTGCCAC	Reverse
CRISPR/Cas9		
OsU3pro(Hind III)F	CCCAAGCTTAAGGAATCTTTAAACATACGA	Forward
gRNA:ter(XbaI)R	TGCTCTAGAAAAACAAAAAAGCACCGACTCGGTGC	Reverse
Os_K18_f_R_5	CCGTGACAACAGGCAGGTGAGCCACGGATCATCTGCA	Reverse
Os_K18_sgRNA_F_5	TCACCTGCCTGTTGTCACGGGTTTTAGAGCTAGAAATAGC	Forward
Protein expression		
CCR10_F_Inf(BamH I)	CAAATGGGTCGCGGATCCATGTCGTCCAACAATTCCA	Forward
CCR10_R_Inf(BamH I)	GAGCTCGAATTCGGATCCCTACAAACATGCACGCTGT	Reverse

Table 3. Substrate specificity of OsCCR10

Enzyme activity	OsCCR10
Coumaroyl-CoA	+
Feruloyl-CoA	+
Sinapoyl-CoA	–

Table 4. Lignin contents in *OsCCR10* overexpressing transgenic plants

Sample (mg g^{-1} cell wall)	Leaf	Stem	Root
NT	6.23 ± 0.18	7.14 ± 0.69	2.25 ± 0.15
GOS2::OsCCR10-#8	7.35 ± 0.09	8.22 ± 1.28	5.48 ± 0.69
GOS2::OsCCR10-#9	8.39 ± 0.28	8.54 ± 1.07	3.34 ± 0.01
GOS2::OsCCR10-#13	8.95 ± 0.81	7.43 ± 0.69	5.56 ± 0.62
RCc3::OsCCR10-#2	5.16 ± 0.46	6.34 ± 0.58	5.6 ± 0.4
RCc3::OsCCR10-#16	5.76 ± 0.05	6.59 ± 0.27	8.24 ± 0.41
RCc3::OsCCR10-#27	5.22 ± 0.23	7.47 ± 1.11	10.46 ± 0.33

Figure 1. The lignin biosynthesis pathway in plant

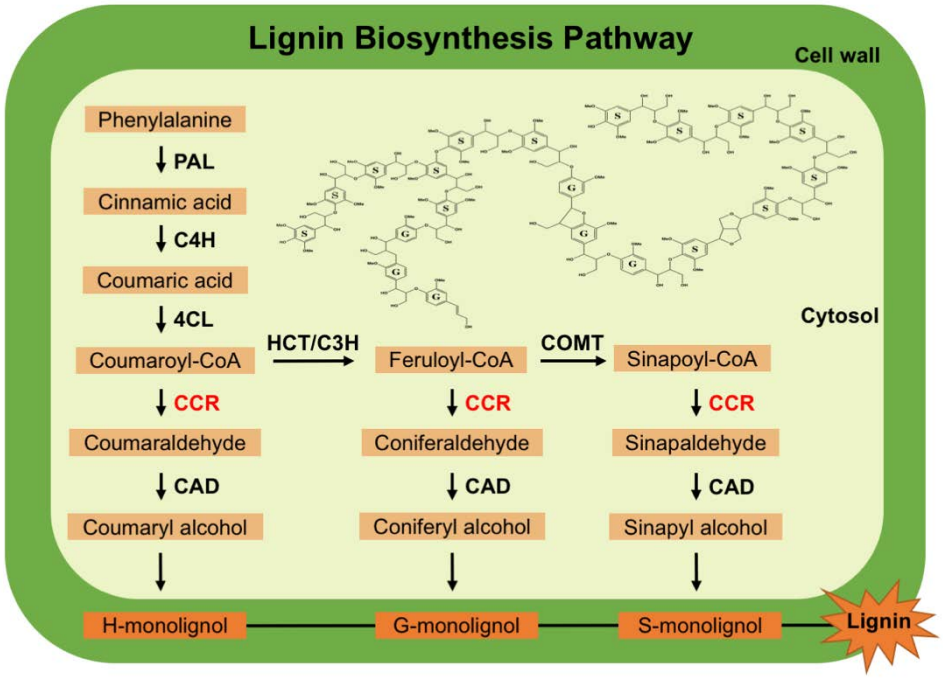


Figure 2. Involvement of *OsCCR10* in *OsERF71*-mediated lignification

(A) Scheme diagram of lignin-mediated drought response mechanism.

(B) Expression level of *OsCCR10* in *ERF71* overexpressing plants.

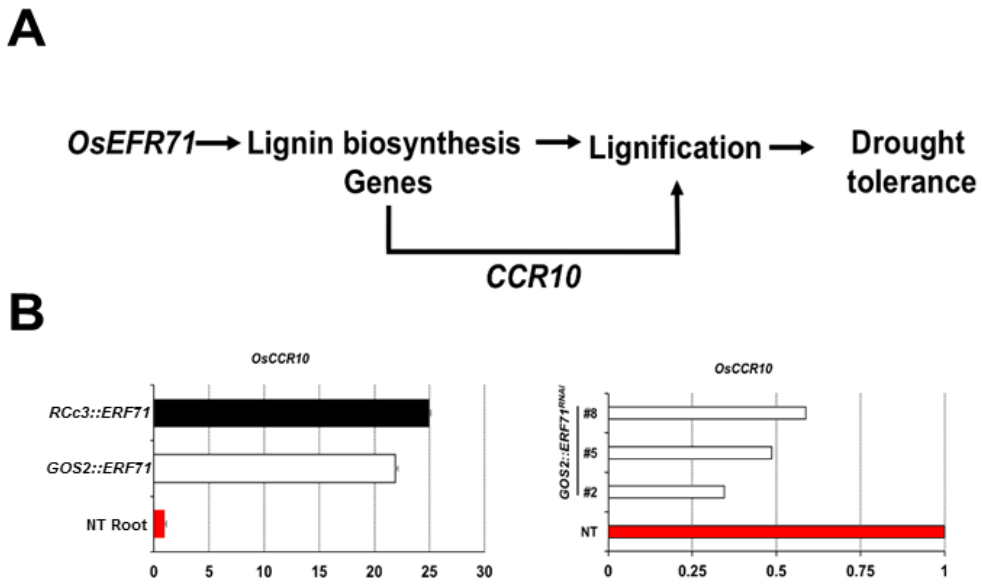


Figure 3. Phylogenetic analysis of *CCRs*

Phylogenetic analysis of *OsCCRs* and 4 characterized CCRs from *Arabidopsis thaliana* and *Zea Mays* was performed using CLC workbench software (http:://www.qiagenbioinformatics.com/products/clc-genomics-workbench). The neighbor-joining tree was built using full-length amino acid sequences of the CCR proteins. Boot strap value was shown for each node. *Arabidopsis thaliana CCRs* (*AtCCR1*, *AtCCR2*); *Zea Mays* CCRs (*ZmCCR1*, *ZmCCR2*)

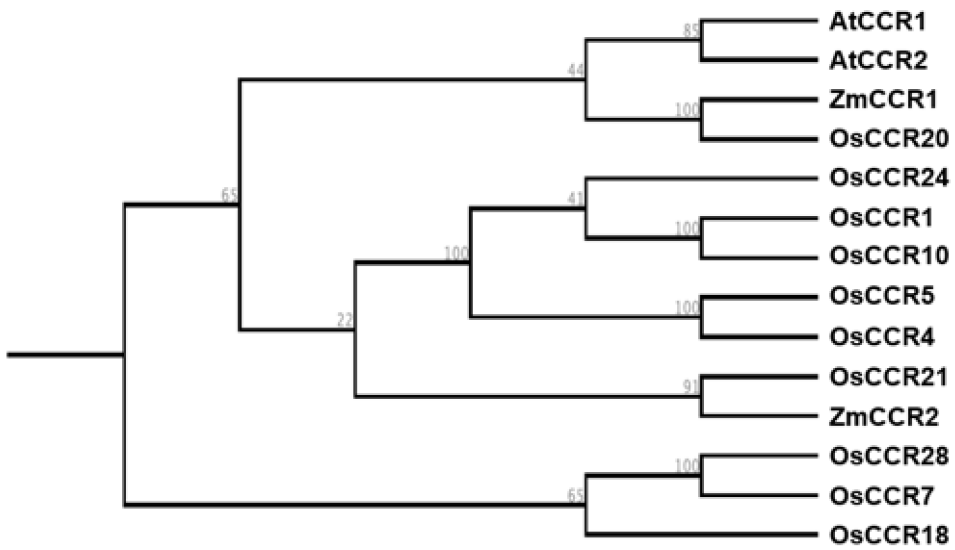


Figure 4. Expression patterns of *OsCCR10* under abiotic stresses

(A) Relative expression levels of *OsCCR10* in response to abiotic stresses. Two-week-old rice seedlings (*Oryza sativa* L. Japonica cv. Ilmi) were exposed to air-drying (drought), 100 μM ABA (ABA), 400 mM NaCl (salt) and 4°C (cold). Leaves and roots of rice plants were harvested at indicated time point after treatment. Rice *UBIQUITIN1* (*OsUBI1)* was used as internal control for normalization Data represent mean value + standard deviation (SD) (n=3).

(B) Relative expression levels of *OsCCR10* in rice plants (*Oryza sativa* cv dong-jin) in various tissue at different developmental stages. The samples were sampled in different stages. Rice *UBIQUITIN1* (*OsUBI1)* was used as internal control for normalization Data represent mean value + standard deviation (SD) (n=3). D, Dark; L, Light; L, Leaf; BH, before heading; AH, after heading; R, Root; C, cotyledon; S, shoot; FL, flag leaf; F flower.

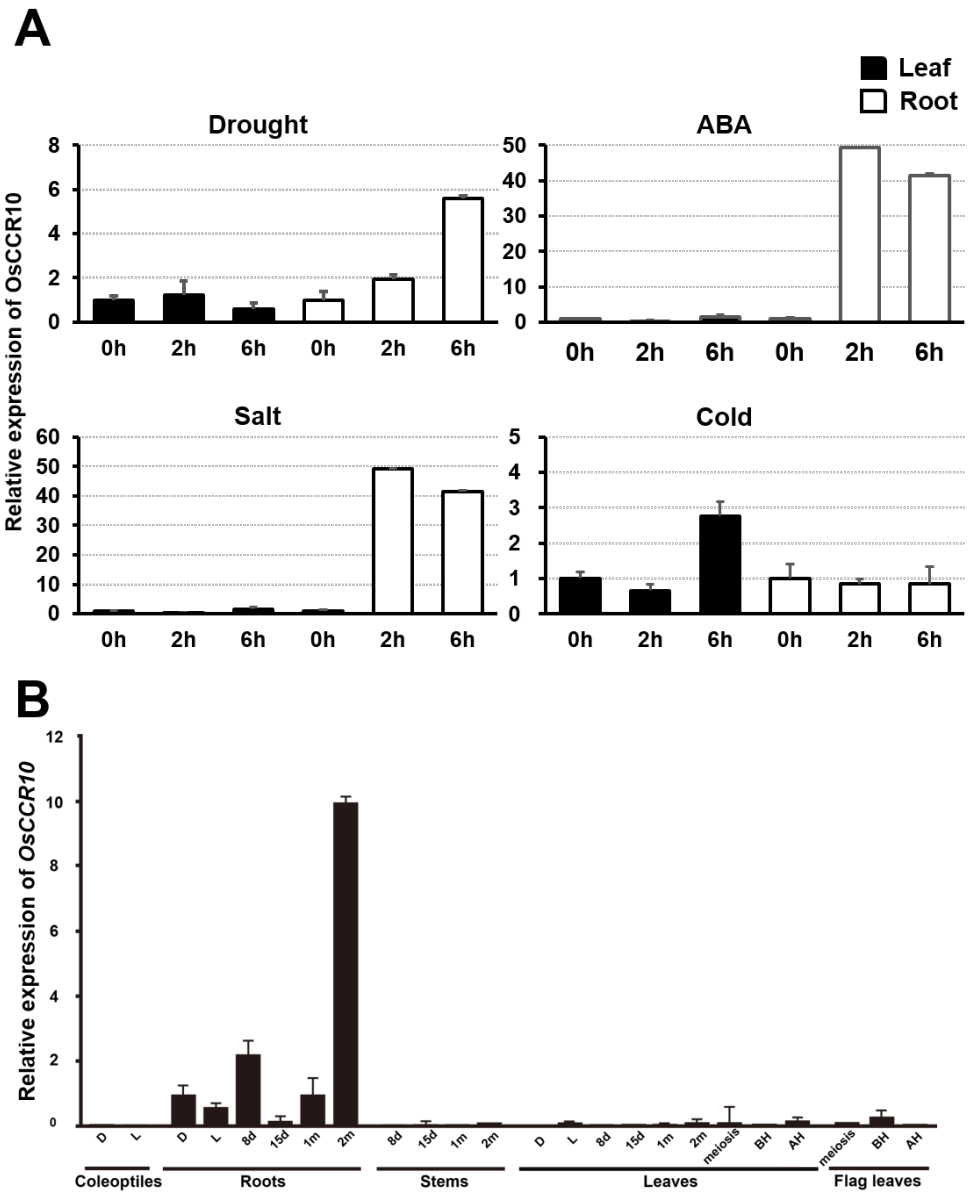

A
Leaf
Root
Drought
ABA
Salt
Cold
Relative expression of OsCCR10
0h
2h
6h
B
Relative expression of OsCCR10
D
L
8d
15d
1m
2m
meiosis
BH
AH
Coleoptiles
Roots
Stems
Leaves
Flag leaves

Figure 5. Subcellular localization of OsCCR10

(A) Schematic diagram of *OsCCR10-GFP* expression construct.

(B) Subcellular localization of OsCCR10 in rice protoplasts. Protoplasts were co-transformed with *OsCCR10-GFP* and indicated subcellular organelle specific markers. Fluorescence of GFP and mCherry was observed using a confocal microscope. Scale bar = 10 μm

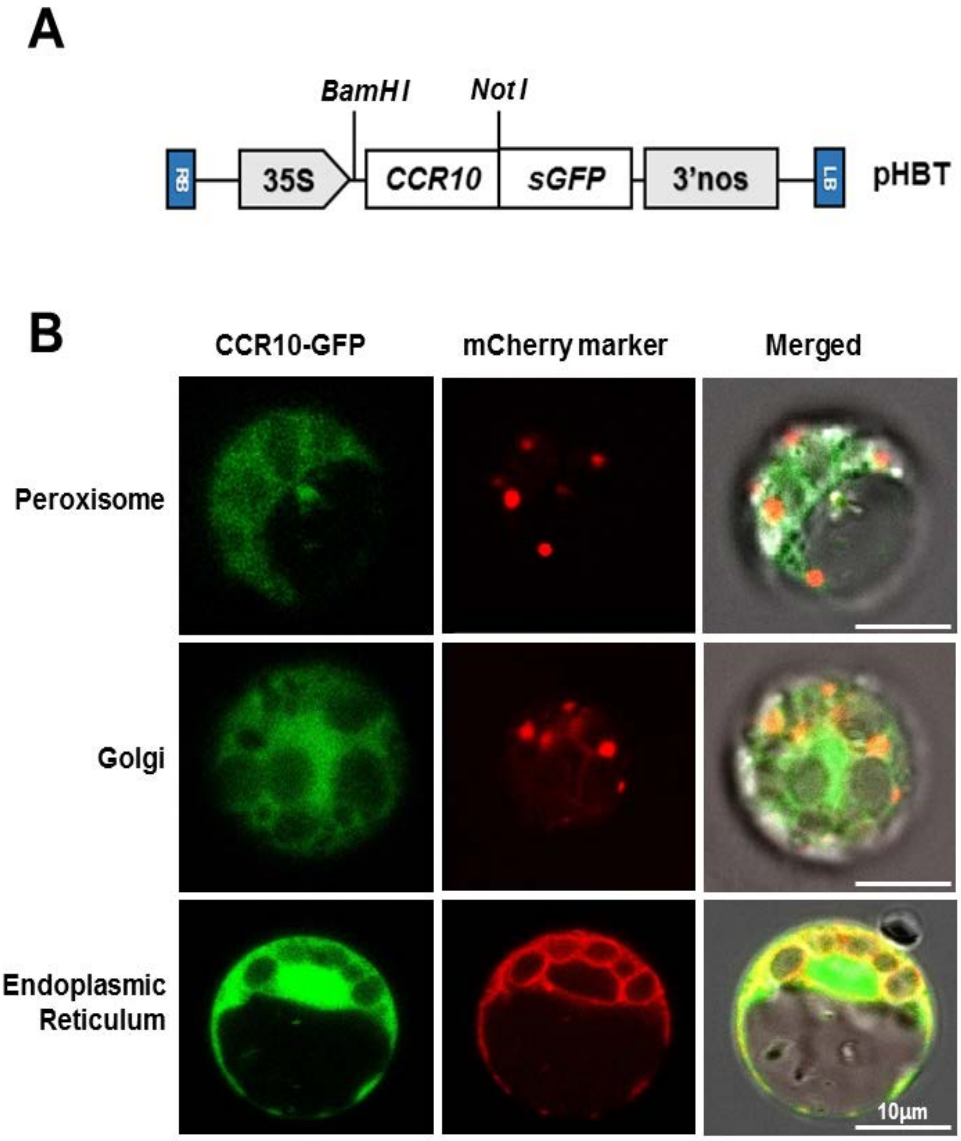

A
BamHI
NotI
RB
35S
CCR10
sGFP
3'nos
LB
pHBT
B
CCR10-GFP
mCherry marker
Merged
Peroxisome
Golgi
Endoplasmic Reticulum
10μm

Figure 6. Generation of *OsCCR10* overexpressing transgenic rice plants

(A) Schematic diagram of *OsCCR10* overexpressing constructs. *OsCCR10* expressed under control of the *GOS2* promoter for whole-body overexpression (*GOS2::OsCCR10*) and *RCc3* promoter for root specific overexpression (*GOS2::OsCCR10*) were cloned into the p700 rice transformation vector.

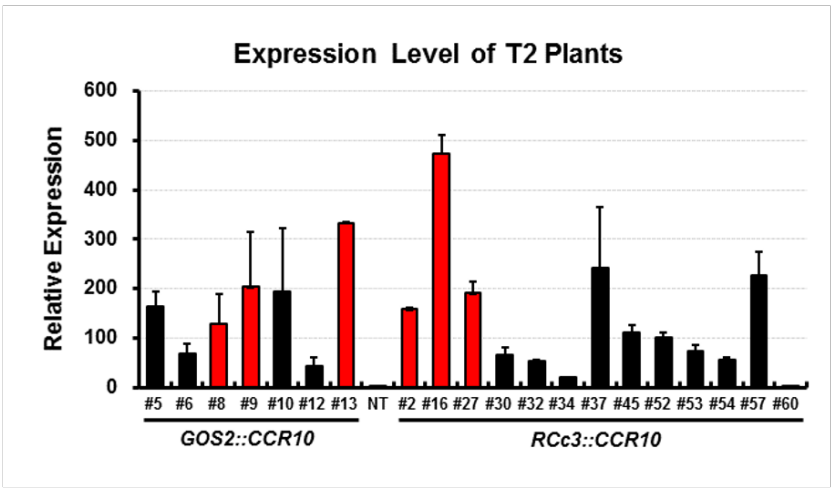
(B) Relative expression of *OsCCR10* was determined in roots of two-week-old non-transgenic (NT) and T_2 generation of *GOS2::OsCCR10* and *RCc3::OsCCR10* transgenic rice plants. *OsUbi1* expression was used as an internal control for normalization. Data represent mean value + standard deviation (SD) (n=3). Red colored lines indicated selected transgenic lines based on expression levels.

(C) Relative expression of *OsCCR10* was determined in shoot and roots of two-week-old non-transgenic (NT) and T_3 generation of selected *GOS2::OsCCR10* and *RCc3::OsCCR10* transgenic rice plants. *OsUbi1* expression was used as an internal control for normalization.

A


P700-GOS2
RB
GOS2
OsCCR10
NOS
Wsi18
sGFP
3'PinII
35s
Bar
NOS
LB
P700-RCc3
RB
RCc3
OsCCR10
NOS
Wsi18
sGFP
3'PinII
35s
Bar
NOS
LB

B


Expression Level of T2 Plants
Relative Expression
600
500
400
300
200
100
0
#5 #6 #8 #9 #10 #12 #13 NT #2 #16 #27 #30 #32 #34 #37 #45 #52 #53 #54 #57 #60
GOS2::CCR10
RCc3::CCR10

C

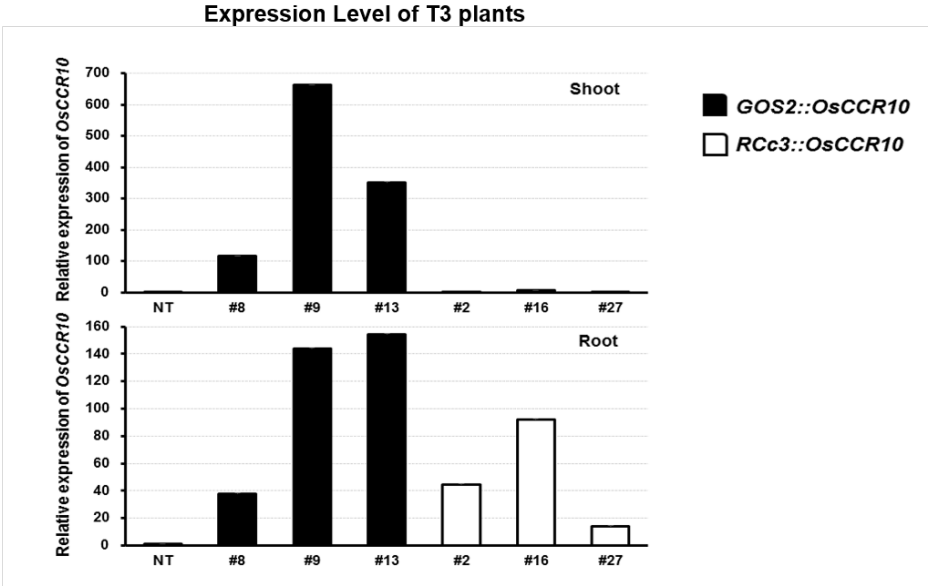

Expression Level of T3 plants
Relative expression of OsCCR10
Shoot
700
600
500
400
300
200
100
0
NT #8 #9 #13 #2 #16 #27
Relative expression of OsCCR10
Root
160
140
120
100
80
60
40
20
0
NT #8 #9 #13 #2 #16 #27
GOS2::OsCCR10
RCc3::OsCCR10

Figure 7. Drought stress tolerance of *OsCCR10* overexpressing transgenic plants in vegetative stage

(A) Drought tolerance of *GOS2::OsCCR10* and *RCc3::OsCCR10* transgenic rice plants was compared with that of non-transgenic plants (NT). Three independent T_3 homozygous *GOS2::OsCCR10* and *RCc3::OsCCR10* transgenic lines and NT control plants were grown in soil for 6 weeks and exposed to drought for 3 days, followed by re-watering for 7 days. Numbers on the images indicate the duration over which drought was imposed and re-watering was applied.

(B) Measurements of soil moisture contents. Soil moisture in the pots was monitored during drought treatment at the indicated time points. Data represent mean value + SD of thirty measurements performed at different locations of soil.

(C) The survival rate of transgenic plants 7 days after re-watering. Each test was performed with 25-30 plants for each transgenic line and 30 plants for NT.

(D) Determination of the photosynthetic viability of transgenic and NT plants under drought conditions. At the indicated time point after exposed to drought stress, chlorophyll fluorescence (F_v/F_m) was measured in the dark at indicated time point after drought treatment using a Handy-PEA fluorimeter. Data represent mean value + SD (n=30).

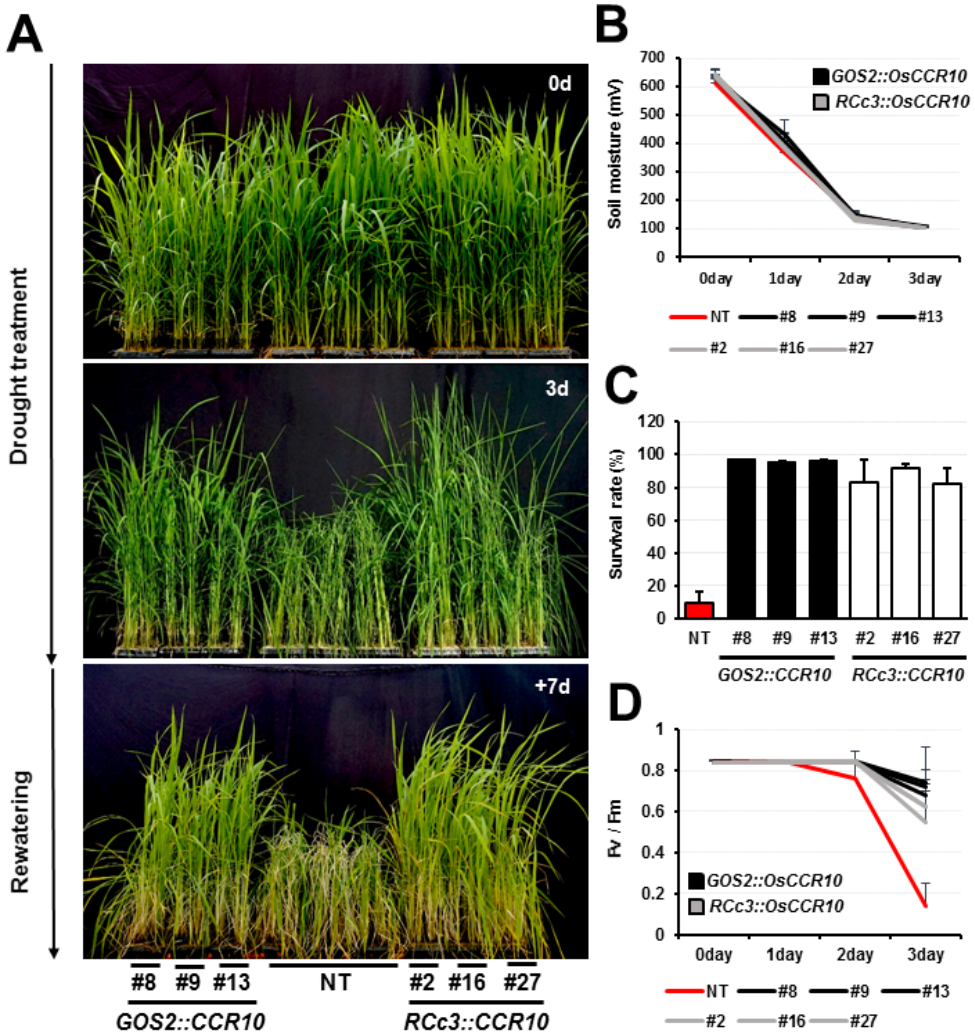
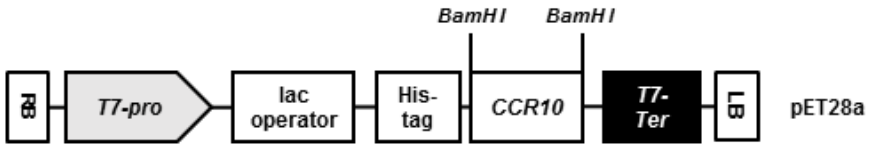

A
Drought treatment
Rewatering
0d
3d
+7d
#8 #9 #13
NT
#2 #16 #27
GOS2::CCR10
RCc3::CCR10
B
Soil moisture (mV)
GOS2::OsCCR10
RCc3::OsCCR10
0day 1day 2day 3day
NT #8 #9 #13
#2 #16 #27
C
Survival rate (%)
NT #8 #9 #13 #2 #16 #27
GOS2::CCR10
RCc3::CCR10
D
Fv / Fm
GOS2::OsCCR10
RCc3::OsCCR10
0day 1day 2day 3day
NT #8 #9 #13
#2 #16 #27

Figure 8. Expression of OsCCR10 recombinant protein

(A) Schematic diagram of a vector expression OsCCR10 recombinant protein. The CDS of *OsCCR10* was cloned into the *pET28a* expression vector to express His-tagged OsCCR10 recombinant protein using infusion cloning through the BamHI restriction enzyme site.

(B) Purification of recombinant OsCCR10 protein expressed in *E. coli*. The His-tagged OsCCR10 was expressed in *E. coli*. The recombinant His-OsCCR10 protein was purified by Ni^{2+}-affinity chromatography. Elution of recombinant OsCCR10 was performed by 40–250mM imidazole in Tris buffer. (M, Molecular weight marker; 1, 40 mM imidazole; 2, 80 mM imidazole; 3, 120 mM imidazole; 4, 160 mM imidazole; 5, 200 mM imidazole; 6, 250 mM imidazole). The eluted protein was analyzed by sodiumdodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

A


BamH I
BamH I
RB
T7-pro
lac operator
His-tag
CCR10
T7-Ter
LB
pET28a

B

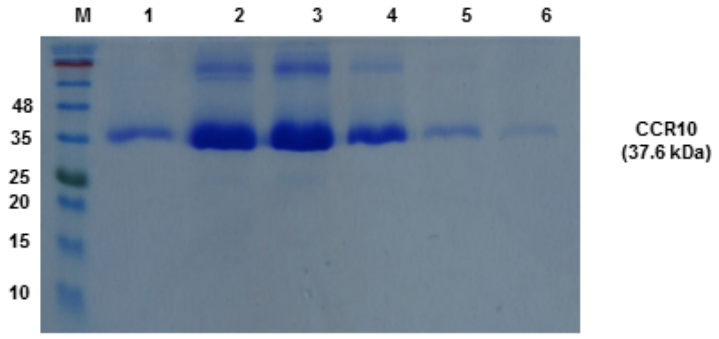
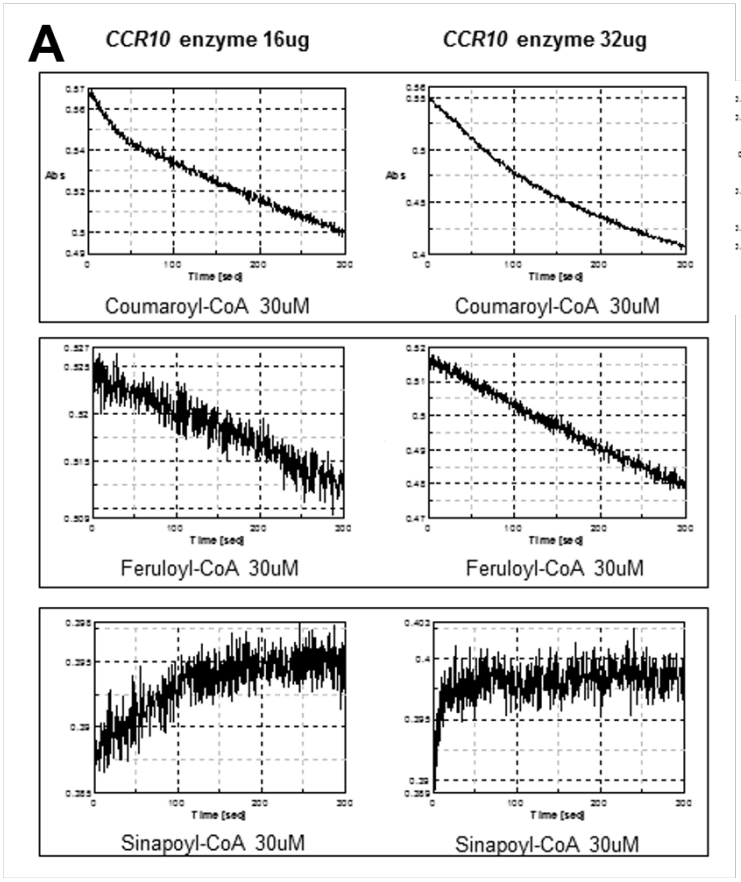
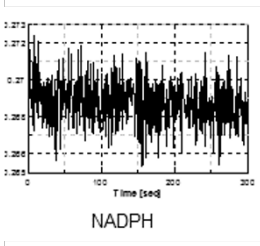

M
1
2
3
4
5
6
48
35
25
20
15
10
CCR10
(37.6 kDa)

Figure 9. Enzyme activity of OsCCR10

(A) *OsCCR10* enzyme activity was checked using coumaroyl-, feruloyl- and sinapoyl-CoA as substrates. A366, representing changes in NADPH, were monitored for 5 minutes. Two different amount of OsCCR10 recombinant protein was tested to determine reaction kinetics. Change of A366 measured value was monitored with NADPH as a negative control.

(B) The schematic diagram of enzyme activity of OsCCR10. OsCCR10 mediates reduction of Coumaroyl-CoA and Feruloyl-CoA but not Sinapoyl-CoA to hydroxycinnamoyl-alcohols using NADPH as a cofactor.


A
CCR10 enzyme 16ug
CCR10 enzyme 32ug
Abs
Time [sec]
Coumaroyl-CoA 30uM
Coumaroyl-CoA 30uM
Feruloyl-CoA 30uM
Feruloyl-CoA 30uM
Sinapoyl-CoA 30uM
Sinapoyl-CoA 30uM


Time [sec]
NADPH

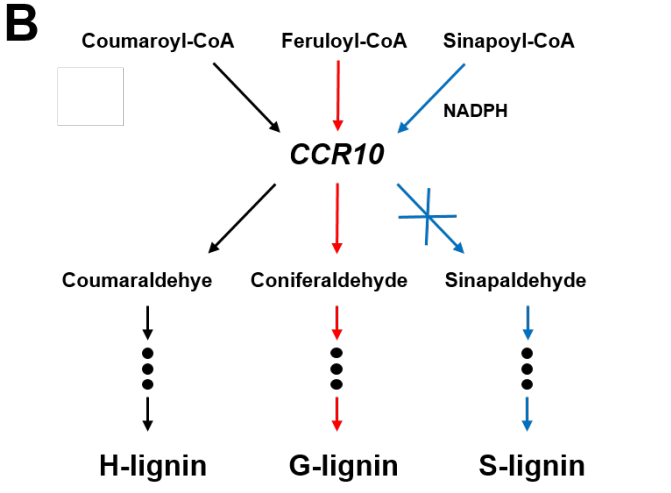
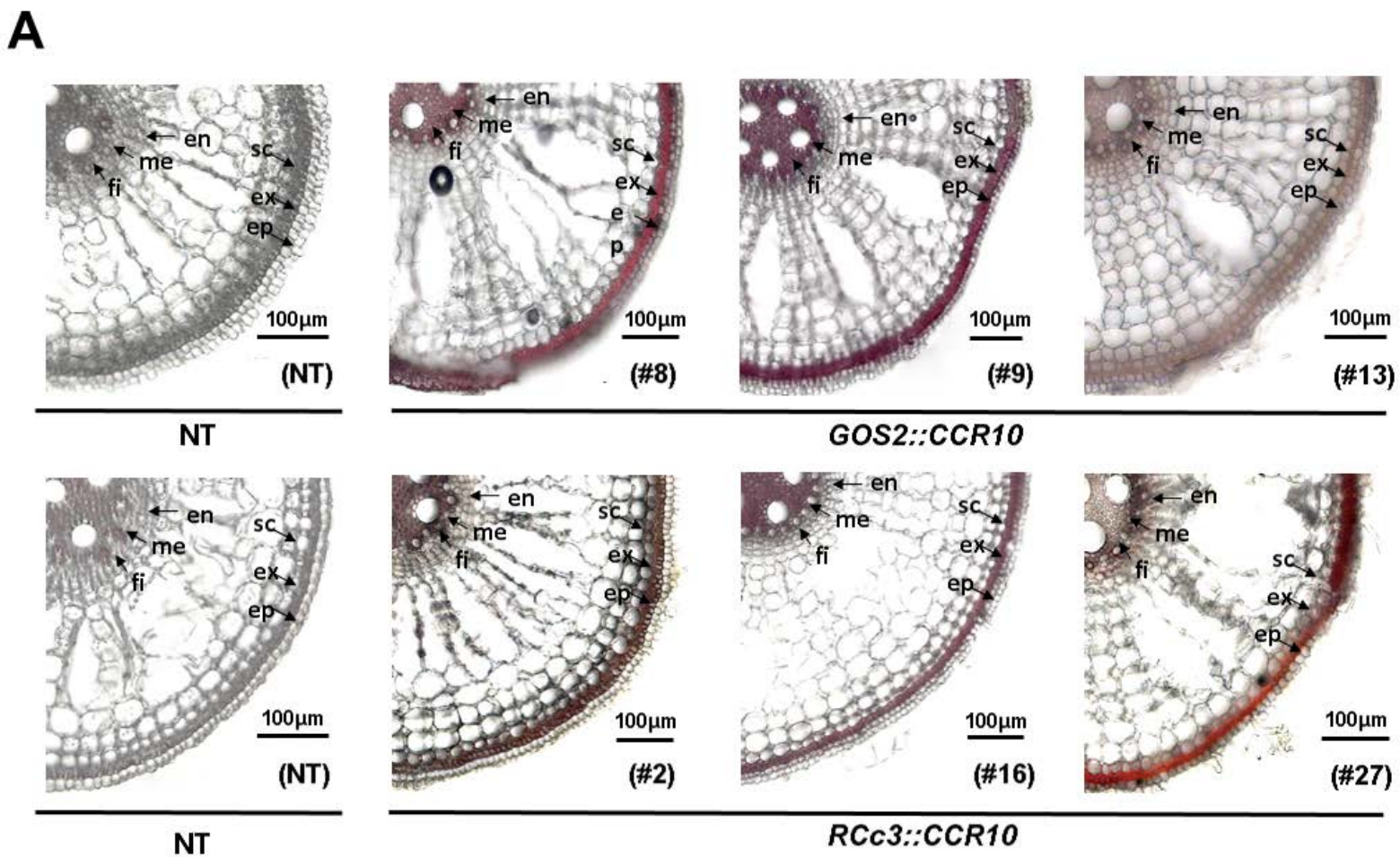

B
Coumaroyl-CoA
Feruloyl-CoA
Sinapoyl-CoA
NADPH
CCR10
Coumaraldehye
Coniferaldehyde
Sinapaldehyde
H-lignin
G-lignin
S-lignin

Figure 10. Lignin staining of *OsCCR10* overexpressing transgenic plants

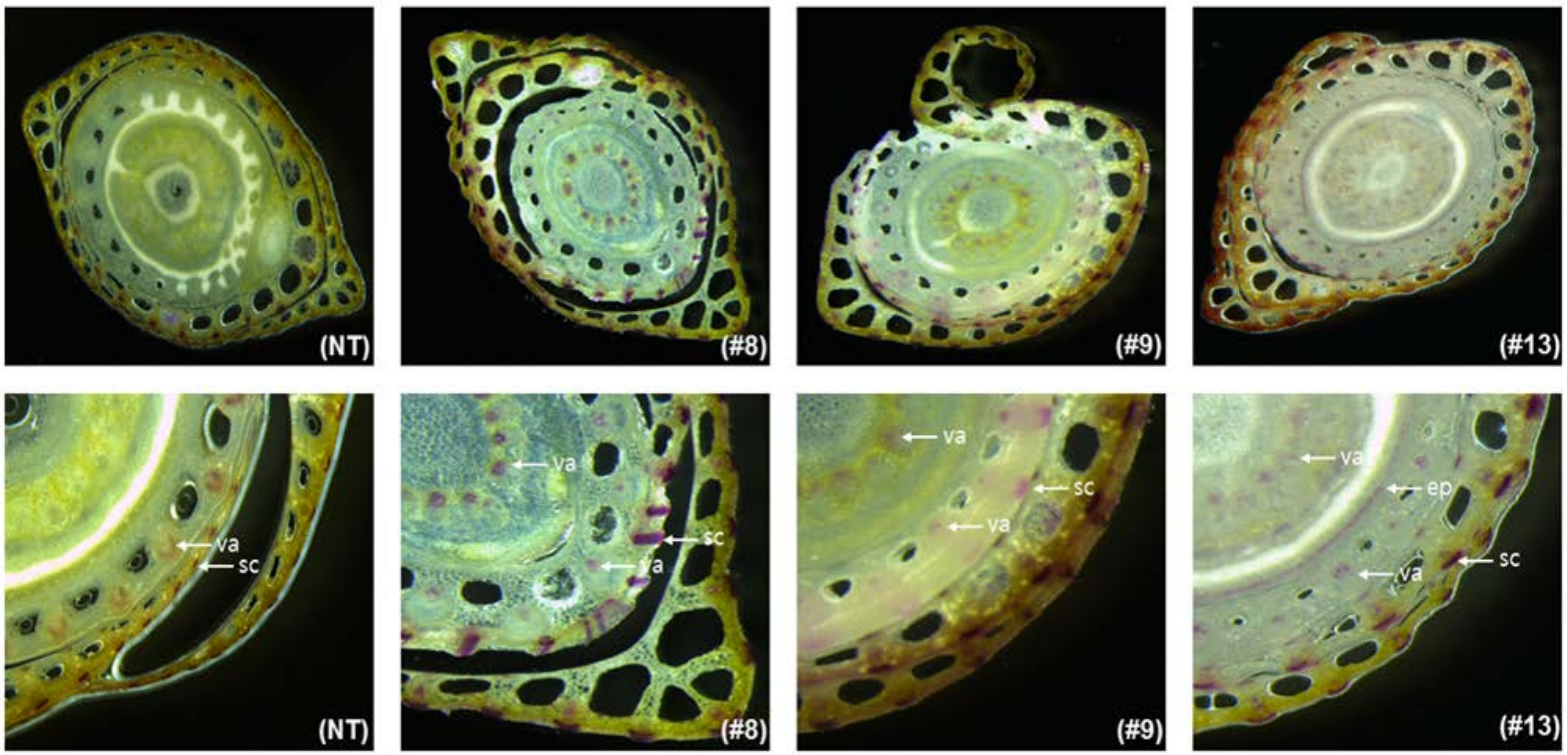
(A) Lignin staining of roots. Transverse hand sections using microtome of two-month-old roots of non-transgenic (NT), *GOS2::OsCCR10*, *RCc3::OsCCR10* transgenic plants were stained with phloroglucinol-HCl. Black arrow heads were used to indicate root cellular structures stained by phloroglucinol-HCl. en, endodermis; me, metaxylem; fi, fiber; sc, sclerenchyma; ex, exodermis; ep, epidermis.

(B) Lignin staining of stems. Transverse hand sections of two-month-old stems of non-transgenic (NT) and *GOS2::OsCCR10* transgenic plants were stained with phloroglucinol-HCl. White arrow heads were used to indicate cellular structures in stems stained by phloroglucinol-HCl. va, vascular bundles; ep, epidermis; sc, sclerenchyma tissues.

(C) Lignin staining of leaves. Transverse hand sections of two-month-old leaves of non-transgenic (NT) and *RCc3::OsCCR10* transgenic plants were stained with phloroglucinol-HCl. White arrow heads were used to indicate cellular structures in stems stained by phloroglucinol-HCl. va, vascular bundles; ep, epidermis; sc, sclerenchyma tissues.


A
en
me
fi
sc
ex
ep
e
p
100µm
(NT)
(#8)
(#9)
(#13)
NT
GOS2::CCR10
(NT)
(#2)
(#16)
(#27)
NT
RCc3::CCR10

B



C

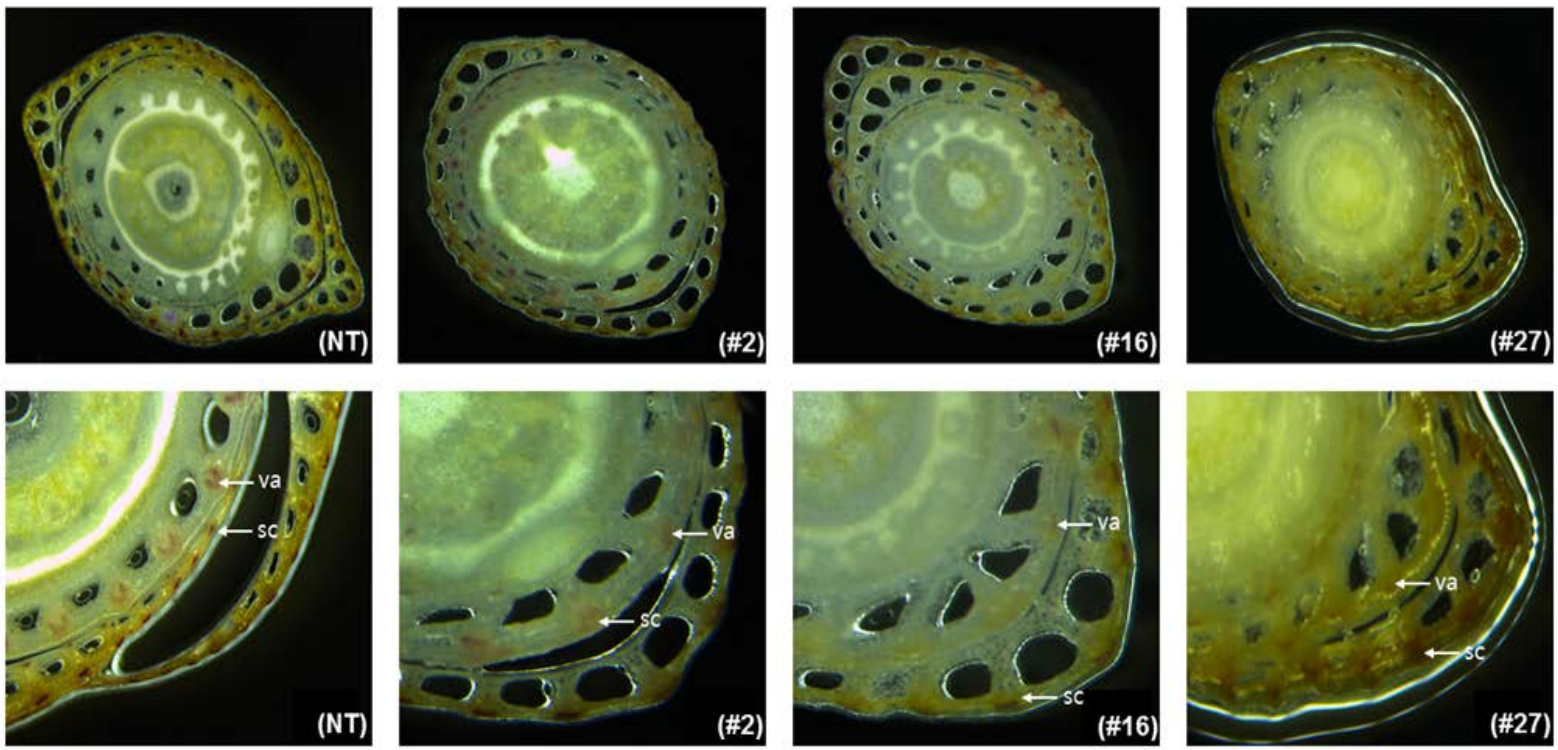
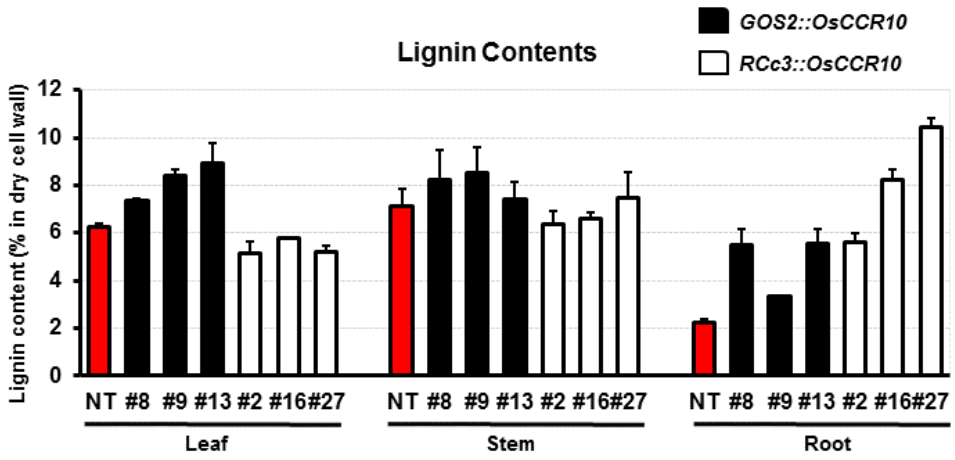


Figure 11. Lignin quantification in *OsCCR10* overexpressing transgenic plants

Lignin contents were quantified in leaves, roots and shoots of Two-month-old non-transgenic (NT), *GOS2::OsCCR10* and *RCc3::OsCCR10* transgenic plants. Data represent mean +SD (n=2).



Discussion

Here, it was found that overexpression of *OsCCR10* enhances drought tolerance. Drought stress at the vegetative stage caused negative effects on chlorophyll biosynthesis and photosynthetic capacity and leads to a significant reduction in plant growth and productivity (Abid et al., 2016; Wada et al., 2019). Therefore, it is crucial to understand drought tolerance mechanisms in plants and identifying key players in drought tolerance pathway. Here, I found that overexpression of *OsCCR10* enhances drought tolerance. The *OsCCR10* overexpressing transgenic plants showed decreased and delayed drought-induced damage in comparison to the non-transgenic (NT) control plants and better recovery after re-watering (Fig. 7A). They also maintained photosynthesis efficiency under drought conditions (Fig. 7D). It has been shown that lignin biosynthesis increased under drought conditions. When drought stress was stimulated, the lignin content was significantly up-regulated in the stem of *Eucalyptus urograndis* and the stem apical region of *Eucalyptus globulus* (Mourasobcazk et al., 2011). It is obvious that cell wall modification is important in adapting cells and organs in plants to water-deficit and osmotic stress (Moore et al., 2008). As a water stress situation such as drought occurs, plants adapt to the stress by accumulating phenolic compounds and accelerating lignification in cell wall (Moore et al., 2008).

Lignin is a biopolymer, composed of p-coumaryl-, coniferyl- and sinapyl-alcohol monomers, which makes cell walls stiffer. Lignin biosynthesis pathway begins with phenylalanine, that's one of the phenolic compounds (Fig. 1) (Miedes et al., 2014). It was founded that enzymes related with lignification were up-regulated when exposed to water stress in white clover (*Trifolium repens* L.) (Lee et al., 2007). Cinnamoyl-CoA reductase (CCR) and cinnamyl alcohol dehydrogenase (CAD) are the final two enzymes functioning in the monolignols biosynthesis pathway (Thevenin et al., 2011). Lignin biosynthesis is complicated process and is regulated by diverse conditions and

stresses (Cabane et al., 2012). The level of *CCR1* and *CCR2* in root elongation zone were increased in maize under water stress conditions, and concluded that lignin biosynthesis controls plant growth under water stress (Fan et al., 2006).

CCR (Cinnamoyl-CoA Reductase) is believed to play an important function in lignin biosynthesis. CCR is responsible for reduction of the CoA ester to aldehyde in monolignol biosynthesis (Boerjan et al., 2003). The previous study (Fan et al., 2006) reported that water deficit stress induced the expression of the *CCRs* as well as lignin biosynthesis in maize. Additionally, the study of *leucaena leucocephala* showed that water deficit treatments induced significant deposition of lignin (Srivastava et al., 2015). It is also suggested that, in addition to *CCR*, other lignin biosynthesis enzymes are associated with drought stress tolerance mechanisms (Chazen and Neumann et al., 1994; Lee et al., 2007). I found here that overexpression of *OsCCR10* is sufficient to increase lignin content in rice plants (Fig. 10, 11). Especially, lignin levels in the *OsCCR10* overexpressing plants were higher in sclerenchyma tissues and fiber in roots compared with NT plants (Fig. 10A). These findings are supported by previous study showing that CCR enzyme are responsible for accumulation of lignin in white popinac (*leucaena leucocephala)* (Srivastava et al., 2015). Since, CCR activity is usually low in plants in normal conditions, stress-inducible regulation of CCR could be a significant control point to regulate metabolite flow towards lignin biosynthesis (Lacombe et al., 1997).

Phenylalanine reacts with many enzymes and turns into lignin (Ramamurthy et al., 2000). Lignin synthesized from phenylalanine is a biopolymer which is composed of H, G and S monomers, these biopolymers promotes plant cell walls strengthen, rigidity, hydrophobic properties and enhance mineral transport through vascular bundles (Boerjan et al., 2003; Liu et al., 2018; Ramamurthy et al., 2000). CCR enzyme functions as a reductase which catalyze hydroxycinnamoyl-CoA (coumaroyl-, feruloyl- and sinapoyl-CoA) to hydroxycinnamoyl-aldehyde (coumar-, conifer- and

sinap-aldehyde) (Piquemal et al., 1998; Jones et al., 2001; Goujon et al., 2003; Kawasaki et al., 2006; Leple et al., 2007; Wadenback et al., 2008; Zhou et al., 2010). It has been reported that each CCR has different substrate preference. Many CCRs, such as *Medicago* CCR1, prefer feruloyl- and sinapoyl-CoAs as substrates (Lauvergeat et al., 2001; Li et al., 2005; Goffner et al., 1994). Maize ZmCCR1 recombinant protein preferred p-coumaroyl-CoA as a substrate (Tamasloukht et al., 2011). In case of *Arabidopsis*, CCR2 shows sensible activity with feruloyl- and sinapoyl-CoAs (Lauvergeat et al., 2001). Therefore, determining substrate preference of CCR is required to understand lignification mechanism in plants. It was found here that *OsCCR10* has reductase activity (Fig. 9) and prefers coumaroyl- and feruloyl-CoA which are precursors of H- and G- monolignol (Fig. 9A). These results suggest that drought-inducible expression of *OsCCR10* could mediate accumulation of H- and G-monolignol in plants. Since enzyme assay data of OsCCR10 have shown that OsCCR10 catalyzes coumaroyl- and feruloyl-CoAs, further study is needed to check monolignol changes through Pyrolysis-GC/MS analysis in overexpression plants.

It was found that *OsCCR10* overexpression increased the plant lignin content (Fig. 10, 11). Interestingly, lignin levels in the *OsCCR10* overexpressing plants were higher in metaxylem, fiber and exodermis of the root compared to NT plants (Fig. 10A). In case of stem, vasculature bundles and sclerenchyma tissues of the *GOS2::OsCCR10* transgenic plants were highly lignified (Fig. 10 B, C). Plant lignification has been known to occur in differentiating xylem tissues and inter fibers in stems and roots (Lacombe et al., 1997; Goujon et al., 2003; Tamasloukht et al., 2011). Thus, tissue specific accumulation of lignin in OsCCR10 overexpressing plants could be explained by tissue-specific expression of other lignin biosynthesis gene or distribution of substrates.

In conclusion, I found that overexpression of the *OsCCR10* gene increases lignin contents which in turns confers drought tolerance in rice. This study suggests that

understanding the function of lignin biosynthesis genes in rice is important to develop drought tolerant crops in crop biotechnology.

Conclusion

In conclusion, I found that *OsCCR10* has an important role for drought tolerance by regulating drought-inducible lignin biosynthesis. In this study, we explored the functions of *OsCCR10* in lignin biosynthesis and drought stress tolerance in rice. OsCCR10 is cytoplasmic protein responsible for reduction of hydroxycinnamoyl-CoA to hydroxycinnamoyl-aldehyde in lignin biosynthesis pathway. *OsCCR10* expression is induced by drought stress, and overexpression of *OsCCR10* is sufficient to increase lignin contents and drought tolerance in plants. This study will provide a strong background for genetic improvements of drought tolerance in rice.

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Abstract in Korean

가뭄 저항성 메커니즘에 연관된 리그닌 생합성 유전자, *OsCCR10* 의 규명

최서원

서울대학교 국제농업기술대학원 국제농업기술학과

지도교수 김 주 곤

가뭄은 식물의 생리학적, 생화학적 및 분자적 변화를 유도하는 일반적인 환경적 제한이다. 그리고 가뭄은 작물 수확량의 막대한 감소를 초래할 수 있다. 그러므로 가뭄 내성의 분자 메커니즘을 이해하는 것은 작물생명공학에 있어 중요한 일이다.

이번 연구에서 우리는 리그닌 생합성 과정 중 hydroxycinnamoyl-CoA 가 hydroxycinnam-aldehyde 로 전환되는 환원제 역할을 하는 *Cinnamoyl-CoA reductase10 (OsCCR10)* 를 과발현 시킨 식물체가 쌀 조직의 리그닌 축적을 증가시켜 가뭄 저항을 향상시키는 것에 대해 알아보았다.

OsCCR10 의 과발현은 비 형질 전환 식물에 비해 생장기에서 가뭄 저항성을 증가시켰다. 또한 효소로써의 작용을 확인하기 위해 전구 물질들과의 상호 반응을 통해 *OsCCR10* 의 활성을 측정하였다. 그리고 리그닌 염색을 통해 표피와 유관속 조직을 포함한 외부 세포층에서 증가된 리그닌화를 유도하였음을 육안으로 확인하였으며, 이는 세포벽 리그닌이 축적된 영역에 상응한다. 정성적인 실험을 뒷받침 하기위하여 우리는 식물의 리그닌 함량을 정량화했다. 과발현 식물체는 총 리그닌 함량에서 높은 축적을 보여 주었다.

OsCCR10 을 과발현 시킨 식물체는 전구 물질들에 대하여 기질 특이적인 반응을 보였다. 이렇게 다른 기질들과 반응 함으로써 리그닌 생합성을 조절하고 리그닌을 축적하여 가뭄에 대한 내성을 증대시킨다는 것을 다양한 실험등을 통하여 밝혀냈다.

키워드 : 가뭄, 벼, CCR, 리그닌, 뿌리, 비생물적 스트레스, 가뭄저항성

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어린시절부터 막연하게 과학자의 꿈을 꾸던 저는 대학원이라는 곳을 통하여 한 걸음씩 나아가게 되었습니다. 여기까지 오는 과정동안 저에게 많은 힘과 도움을 주신 많은 분들 덕분에 이렇게 논문을 마무리 할 수 있는 기회가 있었다고 생각합니다. 부족한 저를 응원해주신 모든 분께 감사의 인사를 전하고자 합니다.

먼저 연구하고 공부할 수 있는 좋은 환경과 기회를 주신 김주곤 교수님께 진심으로 감사드립니다. 마지막까지 믿고 이끌어주신 교수님께서 해주신 말씀을 기억하며 앞으로 한걸음씩 더 나아가는 연구자가 되도록 노력하겠습니다. 또한, 항상 많은 조언을 해주시며 좋은 말씀을 해주신 강진호 교수님, 정춘균 교수님, 서장균 교수님께도 깊은 감사의 인사를 드립니다.

처음 입학하였을 때 부족한 저에게 실험을 알려주시며 많은 일들을 함께 하시고 챙겨주신 저의 사수 이동근 박사님께 감사드립니다. 항상 열정가득한 모습과 저를 옆에서 차근차근 챙겨주신 심재성 박사님께도 감사의 인사를 드립니다. 또한 밤마다 좋은 말씀을 해주시고 챙겨주시던 정필중 박사님께 감사드립니다. 필드에서 항상 멋진 모습을 보여주시고 항상 신경써주신 김연식 박사님, 운동을 같이하고 실험뿐 아닌 평소에 옆에서 많은 도움을 준 승운이형, 외국에서도 자주 연락을 주며 응원해주던 마크형, 항상 지나가며 좋은 말을 해주던 알리박사님에게도 감사 드립니다. 더운 온실에서 식물이 잘 자랄 수 있도록 신경써주신 김정숙 선생님, 조직 배양을 자세히 알려주시며 옆에서 응원해주신 이세남 선생님, 실험실의 모든 업무를 책임지고 도와주신 이미진 선생님께도 감사를 드립니다.

석사 과정동안 또 다른 조력자들이 있습니다. 귀찮을 수 있는 일들을 도와준 소윤이, 졸업 한 후에도 옆에 있는 것처럼 항상 본인 일처럼 도와주던 성환이, 항상 같은 모습과 같은 반응으로 대답해주며 응원해주던 주희, 끝까지 본인 일을 열심히 하며 묵묵히 옆에서 도와주던 주연이에게 항상 감사합니다. 앞으로 더욱 더 발전하고 성장할 신희와 세은이도 고마움을 전합니다. 그리고 옆에서 응원을 해주던 보람누나, 수정누나, 시원이, 경재, 명휘, 원기, 재인이, 성민이, 누리, 대헌이, 문주에게도 감사합니다.

필드에서 처음 인사를 하며 매번 맛있는걸 사주시며 조언을 해주시던 태영이 형, 짧은 시간이었지만 실험실에서 처음 연락을 드렸던 정하린 박사님께도 감사함을 전하고 싶습니다.

마지막으로 항상 옆에서 저를 믿고 응원해주신 부모님에게도 사랑한다는 말과 감사인사를 드립니다. 형과 형수님께도 석사과정을 응원해주셔서 감사합니다.

이외에도 많은 분들께 감사의 인사를 드립니다. 많은 분들의 응원을 잊지않고 앞으로 더 낮은 자세로 더욱 더 성장하며 발전하는 연구자로 성장하겠습니다. 감사합니다.