CROWN ROOTLESS1 binds DNA with a relaxed specificity and activates OsROP and OsbHLH044 genes involved in crown root formation in rice

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SUMMARY

In cereals, the root system is mainly composed of post-embryonic shoot-borne roots, named crown roots. The CROWN ROOTLESS1 (CRL1) transcription factor, belonging to the ASYMMETRIC LEAVES2-LIKE/LATERAL ORGAN BOUNDARIES DOMAIN (ASL/LBD) family, is a key regulator of crown root initiation in rice (Oryza sativa). Here, we show that CRL1 can bind, both in vitro and in vivo, not only the LBD-box, a DNA sequence recognized by several ASL/LBD transcription factors, but also another not previously identified DNA motif that was named CRL1-box. Using rice protoplast transient transactivation assays and a set of previously identified CRL1-regulated genes, we confirm that CRL1 transactivates these genes if they possess at least a CRL1-box or an LBD-box in their promoters. In planta, ChIP-qPCR experiments targeting two of these genes that include both a CRL1- and an LBD-box in their promoter show that CRL1 binds preferentially to the LBD-box in these promoter contexts. CRISPR/Cas9-targeted mutation of these two CRL1-regulated genes, which encode a plant Rho GTPase (OsROP) and a basic helix–loop–helix transcription factor (OsbHLH044), show that both promote crown root development. Finally, we show that OsbHLH044 represses a regulatory module, uncovering how CRL1 regulates specific processes during crown root formation.

Keywords: ASL/LBD transcription factor, CRL1, DNA binding domain, crown root, development, rice, gene regulatory network, Oryza sativa.

INTRODUCTION

The plant-specific ASYMMETRIC LEAVES2-LIKE/LATERAL ORGAN BOUNDARIES DOMAIN (ASL/LBD) transcription factor family originated in streptophyte algae and evolved to control essential functions in land plants (Chanderbali et al., 2015; Coudert et al., 2013a; Coudert et al., 2013b;
Post-embryonic roots develop either from roots, to form lateral roots, or from other organs, usually stems, to form adventitious roots such as crown roots in cereals (Gonin et al., 2019). Despite these major differences between the root and shoot organs from which they originate, the genetic pathways that regulate lateral root formation in Arabidopsis and crown root development in cereals share some similarities (Coudert et al., 2013a; Coudert et al., 2013b; Hochholdinger et al., 2004; Orman-Ligeza et al., 2013). In both cases, local auxin accumulation induces the degradation of auxin/indole-3-acetic acids (AUX/IAAs) (Bian et al., 2012; Grones & Friml, 2015; Sauer & Kleine-Vehn, 2011; Tomas et al., 2013; Xia et al., 2012) and the activation of specific AUXIN-RESPONSIVE FACTORS (ARFs), which leads to the activation of ASL/LBD transcription factors involved in the initiation of new root primordia (Li et al., 2015; Liu et al., 2018; Zenser et al., 2001). In Arabidopsis, AtASL18/LBD16, AtASL20/LBD18, AtASL16/LBD29, and AtASL24/LBD33 are essential for lateral root initiation (Berckmans et al., 2011; Feng et al., 2012; Goh et al., 2012; Lee et al., 2009; Lee et al., 2013; Lee et al., 2015; Okushima et al., 2007). In cereals, the ASL2/LBD transcription factor CROWN ROOTLESS1 (CRL1) in rice (Oryza sativa) and its ortholog ROOTLESS CONCERNING CROWN AND SEMINAL ROOTS (RTCS) in maize (Zea mays) control crown root development (Inukai et al., 2005; Liu et al., 2005; Okushima et al., 2007; Taramino et al., 2007).

Only a few direct targets of ASL/LBD transcription factors have been characterized in Arabidopsis lateral root development (Berckmans et al., 2011; Lee et al., 2009; Lee et al., 2013; Okushima et al., 2007). In rice, a transcriptomic analysis identified 277 genes induced early after CRL1 expression (Coudert et al., 2015). Among these genes, QUIESCENT-CENTER-SPECIFIC HOMEOBOX (QHB), an ortholog of WUSCHEL-RELATED HOMEOBOX 5 (AtWOX5) that plays an important role in quiescent center differentiation, maintenance of the root apical meristem (Sarkar et al., 2007), and quiescent center specification during lateral root development (Goh et al., 2016), was retrieved.
alone was not able to bind N18. A super-shift experiment using a purified rabbit antiserum directed against a specific fragment of 152 amino acids of the C-terminal part of CRL1 outside of the conserved AS2/LOB domain confirmed that the shifted signal observed with MBP-CRL1 was the consequence of an interaction between CRL1 and the N18 DNA probe (Figure S1).

To identify the nucleotide sequences recognized by CRL1, a SELEX experiment was then performed using MBP-CRL1 and the N18 probes. An MBP-polydactyl zinc finger protein (MBP-4ZF) for which the DNA binding sequence is known was used in parallel as a positive control (McNamara, 2002). After 10 rounds of selection, DNA sequences interacting with MBP-CRL1 or MBP-4ZF were cloned and sequenced. The 13-nucleotide sequence known to be specifically recognized by 4ZF was recovered in the 16 clones sequenced, at the exception of a variation of C to T at position 4, thus confirming that the SELEX method was functioning properly and that the fusion with MBP did not affect the DNA binding properties of the 4ZF transcription factor (Figure S2). From 43 distinct sequences isolated using MBP-CRL1, we identified a novel CRL1 binding sequence (CACA[A/C]C), which we named CRL1-box (Figure 1a). Gel shift experiments showed that CRL1 was able to bind not only the CRL1-box but also the LBD-box in vitro, suggesting that CRL1 has a relaxed DNA binding specificity (Figure 1b). In order to evaluate the DNA binding affinity of CRL1 for the LBD-box and the CRL1-box, increasing amounts of unlabeled LBD-box or CRL1-box were added to the binding reaction containing radiolabeled CRL1-box and CRL1 (Figure 1c). The CRL1/CRL1-box complex was strongly and quickly dissociated by increasing excess unlabeled CRL1-box levels from 25-fold to 250-fold. By contrast, excess unlabeled LBD-box was not able to compete efficiently with the CRL1/CRL1-box interaction. These results showed that in vitro CRL1 interacts with the CRL1-box with a higher affinity than with the LBD-box.

CRL1 binds both the CRL1-box and the LBD-box in vivo

To test whether CRL1 can bind to the CRL1-box and the LBD-box in vivo, we took advantage of its positive transcription regulatory activity (Coudert et al., 2015) to perform a transient activation assay in rice protoplasts. First, expression of a CRL1-GFP fusion protein in rice protoplasts confirmed the expected nuclear localization of CRL1 (Figure S3). The ability of CRL1 to transactivate the β-glucuronidase (GUS)-encoding reporter gene placed under the control of a minimal promoter and a tetramer of either the CRL1-box or the LBD-box was then tested in rice protoplasts. Mutated versions of the two boxes in two of their most conserved nucleotides were used as controls (Figure 2a). CRL1 transactivated both CRL1-box- and LBD-box-containing promoters (Figure 2b). These activations

![Figure 1](image_url)
OsROP and OsbHLH044 are direct targets of CRL1

Figure 2. The CRL1-box and the LBD-box allow transactivation by CRL1 in vivo.

(a) Reporter constructs consisted of the GUS gene under the control of a minimal promoter (~47 to 0) and driven by enrichment of native or mutated (m) cis-regulatory sequences (~138 to ~92). Bold nucleotides indicate point mutations in LBD- and CRL1-boxes. Numbers indicate positions relative to the start site of transcription of the GUS gene.

(b) Transactivation of the GUS reporter gene placed under the control of an LBD-box, a CRL1-box, or their corresponding mutated sequences (m) by CRL1 in rice protoplasts. Rice protoplasts were co-transformed with (i) reporter plasmids carrying the GUS reporter gene placed under the control of a minimal promoter and tetramers of an LBD-box, a CRL1-box, and their corresponding mutated sequences, (ii) overexpression vectors without or with the CRL1 gene driven by the CaMV 35S promoter, and (iii) the p2rL7 normalization plasmid (De Sutter et al., 2005) carrying the LUC gene driven by a CaMV 35S promoter. Values represent means ± SE of triplicate experiments. Student’s t-tests were used to compare the obtained relative GUS expression levels. Values were significantly different at \( P = 0.05 \) (*), \( P = 0.01 \) (**), and \( P = 0.001 \) (***)

Figure 3. Block scanning mutagenesis of the CRL1-box using in vivo transactivation assays with CRL1 reveals most important bases in this sequence.

(a) Table of the different mutated DNA sequences of the CRL1-box used in the transient assay in rice protoplasts. Bold indicates nucleotide mutations.

(b) Transactivation of CRL1-box and several mutated motifs by CRL1 in rice protoplasts. Rice protoplasts were co-transformed with (i) reporter plasmids carrying the GUS reporter gene placed under the control of a minimal promoter and tetramers of an LBD-box, a CRL1-box, and its mutated form fused to GUS, (ii) over-expression vectors without or with the CRL1 gene driven by the CaMV 35S promoter, and (iii) the p2rL7 normalization plasmid (De Sutter et al., 2005) carrying the LUC gene driven by a CaMV 35S promoter. Values represent means ± SE of triplicate experiments. Student’s t-tests were used to compare the obtained relative GUS expression levels. Values were significantly different at \( P = 0.05 \) (*), \( P = 0.01 \) (**), and \( P = 0.001 \) (***)

(c) Positive (+) and negative (−) signs indicate the nucleotides of the CRL1-box DNA sequence that positively or negatively influence transactivation by CRL1. Smaller letters indicate less important nucleotides for CRL1 binding activity. Numbers indicate the position of the bases.

decreased or were abolished when the CRL1-box or the LBD-box was mutated. These data confirmed that CRL1 can bind both the CRL1-box and the LBD-box and acts as a transcriptional activator in rice cells.

To know whether this relaxed DNA binding specificity was specific to CRL1, we performed a transient activation assay using the Arabidopsis AS2 transcription factor that was initially used to identify the LBD-box (Husbands et al., 2007). In rice protoplasts, AS2 was also able to transactivate the LBD- and CRL1-boxes, indicating that other ASL/LBD transcription factors than CRL1 can have a relaxed binding specificity for these two DNA sequences (Figure S4).

Block scanning mutagenesis of the CRL1-box was performed to pinpoint the importance of each nucleotide for transactivation by CRL1 (Figure 3). Six mutated variants were used in addition to the CRL1-box (Figure 3a). The same level of transactivation was observed for the CRL1-box and mutated form 3, showing that the two nucleotides in positions 3 and 4 do not affect CRL1 activity (Figure 3a, b). By contrast, we observed a higher transactivation with
the mutated variants 2 and 5, and a lower activation with the variants 1 and 4, suggesting that in the CRL1-box, the second and sixth nucleotides have a negative influence on CRL1 activity whereas the first and fifth nucleotides of the CRL1-box positively influence CRL1 activity (Figure 3a.b). Low GUS activity under the control of the mutated variant 6 suggested that this sequence is not recognized by CRL1. In conclusion, we identified the nucleotides having a positive or negative influence on the CRL1/CRL1-box interaction (Figure 3c).

A set of putative CRL1 target gene promoters are transactivated by CRL1 in rice cells

Previous transcriptome analyses identified 277 genes upregulated 4 hours after CRL1 induction (Coudert et al., 2015). Out of them, we selected six genes encoding regulatory proteins (Table S1) based on (i) their known function in root development, (ii) their expression level after CRL1 induction (Coudert et al., 2015), (iii) the presence in their promoter of either the CRL1-box or the LBD-box sequence, a combination of those, or none of them (Figure 4a). Moreover, expression profiles in an earlier published transcriptomic data set supported the hypothesis that these genes were targets of CRL1 (Lavarenne et al., 2019) (Figure S5). We performed a transactivation assay in rice protoplasts to test this hypothesis (Figure 4b). For each of these six selected putative CRL1 target genes, 1000- to 1500-bp promoter fragments upstream of the predicted transcription start site were fused to a minimal promoter and the GUS-encoding reporter gene. The QHB promoter was used as a positive control, having already been shown to be transactivated by CRL1 in rice protoplasts (Lavarenne et al., 2019). Rice protoplasts were then co-transformed with a promoter-GUS construct, an overexpression vector containing the CRL1 cDNA driven by the cauliflower mosaic virus (CaMV) 35S promoter, and a reference plasmid carrying the Renilla firefly luciferase (LUC) gene. An empty vector without the CRL1 sequence was used as a control. We found that CRL1 transactivates all the tested promoters except pOsHOX17 (Figure 4b), the only gene whose promoter does not contain a CRL1-box or an LBD-box. This suggests that the presence of at least one of the two boxes in the promoter is required for transactivation by CRL1. Since OsHOX17 is induced downstream of CRL1 (Coudert et al., 2015; Lavarenne et al., 2019) (Figure S5), this indicates that OsHOX17 is most probably an indirect target of CRL1.

CRL1 binds the promoters of OsROP and OsbHLH044 in planta

To further investigate whether the observed transactivation by CRL1 in rice protoplasts is due to a direct interaction of CRL1 with the CRL1-box or the LBD-box, we performed ChIP-qPCR assays on the promoters of OsbHLH044 and OsbHLH044, which both contain CRL1- and LBD-boxes. For this purpose, a rice transgenic line expressing a CRL1-IA fusion protein (hereafter named DXCH) under the control of a dexamethasone (DEX)-inducible promoter in the cr1 mutant background was developed. Treatment of the DXCH line with DEX induced the accumulation of CRL1-HA mutant background was developed. Treatment of the DXCH line with DEX induced the accumulation of CRL1-HA fusion protein in nuclear protein extracts of stem bases (Figure S6d); this accumulation started 3 h after DEX treatment and increased until 9 h (Figure S6b). Ten days after DEX treatment, development of crown roots was observed from the stem base of the DXCH line (Figure S6c), showing that CRL1-HA was functional and able to complement the cr1 mutant line. ChIP assays were then performed using stem base samples of the DXCH line with an anti-HA commercial antibody, and immunoprecipitated target DNA

(a) Gene Annotation LBD-box CRL1-box

| Gene       | Annotation | LBD-box | CRL1-box |
|------------|------------|---------|----------|
| OsbHLH044  | bHLH       | +       | CACACC   |
| OsbHOX17   | HD ZIP     | -       | -        |
| OsROP      | RhoGTPase  | +       | CACACC   |
| ROC4       | HD ZIP     | -       | CACACC   |
| OsbHOX34   | HD ZIP     | -       | CACACC and CACACC |

(b)
sequences were detected and quantified by qPCR using primers designed to amplify regions containing the CRL1-box and/or the LBD-box in the OsROP and OsbHLH044 promoters (Figure 5a). Their relative enrichment was calculated by comparing samples obtained 3 h or 9 h after induction of CRL1-HA expression by DEX and the control prior to DEX induction (T0). For both genes, promoter fragments containing a CRL1-box were not enriched after induction of CRL1-HA expression by DEX, whereas the quantity of immunoprecipitated promoter fragments containing only an LBD-box was significantly increased 3 h and/or 9 h after DEX treatment (Figure 5b,c). These data indicate that OsROP and OsbHLH044 are direct target genes of CRL1. Consistently, the expression profiles of OsROP and OsbHLH044 after CRL1 induction show that both genes were induced until 12 h after DEX induction (hai). After 12 hai, OsROP expression was maintained at the same level, whereas OsbHLH044 expression was inhibited, suggesting that they act differentially downstream of CRL1 (Figure S7) (Lavarenne et al., 2019).

**CRL1 target genes OsROP and OsbHLH044 promote crown root formation**

To investigate the roles of OsROP and OsbHLH044 in crown root development, complementary functional approaches were undertaken. First, we generated knockout mutants for OsROP and OsbHLH044 in the rice short cycle cultivar kitaake via CRISPR/Cas9 gene editing. Second, we overexpressed OsROP and OsbHLH044 in cv. Taichung 65 rice plants. Third, these genes were overexpressed in the crl1 mutant (background cv. Taichung 65) to test whether they could complement the crl1 phenotype.

For each gene, three independent homozygous CRISPR/Cas9 lines harboring different knockout mutations shifting or deleting a large part of the open reading frame and without the T-DNA were selected (Figure S8). CRISPR/Cas9-generated loss-of-function mutant lines of OsROP and OsbHLH044 are hereafter named osrop cas and osbhlh044 cas, respectively. After 4 weeks of growth, osrop cas and osbhlh044 cas mutant plants showed a significant reduction of crown root number compared to wild-type (WT) plants (Figure 6a,b). This reduction of the number of crown roots was about 20% in osrop cas lines and 30–60% in osbhlh044 cas lines. A reduction of the number of tillers was also observed in about 20% in the osrop cas line and in 30–50% in the osbhlh044 cas line in comparison with the WT (Figure S9). To test whether osrop and osbhlh044 mutations acted specifically on the crown root number, we measured the crown root number at an early stage of development before tillering. The crown root number was significantly reduced in both osrop cas and osbhlh044 cas mutant lines (Figure S10). This shows that both genes are involved in crown root formation independently of the number of tillers, which suggests that tillering defects observed in later developmental stages might be a consequence of the reduced crown root number. Consistently, OsROP (OsROP_Tc65OE) or OsbHLH044 (OsbHLH044_Tc65OE) overexpression lines in the Taichung 65 genetic background had a significantly increased crown root number (Figure 6c,d). We also observed an increase in tiller number in OsbHLH044_Tc65OE, whereas only one of the three OsROP_Tc65OE lines presented a significant increase of tiller number (Figure S9). When overexpressed in the crl1 mutant background (OsROP_crl1OE and OsbHLH044_crl1OE lines), no significant differences in crown root number at the early stage of development

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**Figure 5.** In plants, CRL1 binds preferentially to the LBD-box in the promoters of OsROP and OsbHLH044.

(a) Positions of primers relative to the transcription start site (arrows: Fw, forward primer; Rev, reverse primer) and of promoter regions amplified (Amp) after ChIP by qPCR in the promoters of OsROP and OsbHLH044. For each amplified region the upper schema indicates the presence and the sequence of the CRL1-box and/or the LBD-box. (b, c) Fold enrichment values as determined by ChIP-qPCR with 3 and 9 h of DEX treatment versus 0 h of DEX treatment (control) of amplicons of OsROP (b) and OsbHLH044 (c). Values were significantly different at P = 0.05 (*), P = 0.01 (**), and P = 0.001 (***).
Figure 6. Mutation and overexpression of OsROP and OsbHLH044 promote crown root formation. (a) Comparison of crown root number between WT and three independent osrop cas or three independent osbhlh044 cas lines. Student’s t-tests were used to calculate the significant differences. Values were significantly different at $P = 0.05$ (*), $P = 0.01$ (**), and $P = 0.001$ (***) N indicates the number of plants. (b) Pictures of WT and three independent osrop cas or three independent osbhlh044 cas lines. (c) Comparison of crown root number between WT and three independent OsROP_TC65OE or two independent OsbHLH044_TC65OE lines. Student’s t-tests were used to calculate the significant differences. Values were significantly different at $P = 0.05$ (*), $P = 0.01$ (**), and $P = 0.001$ (***) N indicates the number of plants. (d) Pictures of WT and three independent OsROP_TC65OE or two independent OsbHLH044_TC65OE lines.
before tillering were observed between crl1OE lines and crl1 plants for both genes although the transgenes were well overexpressed (Figure S11). Altogether, our results indicate that OsROP and OsbHLH044 promote crown root development in rice. However, neither OsROP nor OsbHLH044 was sufficient to restore crown root development in the crl1 background and therefore to complement CRL1 function.

OsbHLH044 REGULATES THE EXPRESSION OF SEVERAL STRESS-RELATED GENES

To determine the manner in which CRL1 target genes contribute to crown root development, we sought to identify OsbHLH044-regulated genes. We analyzed the transcriptional profile of OsbHLH044_crl1OE stem bases in comparison with corresponding null sister control lines (N_OsbHLH044_crl1OE) and identified 39 differentially expressed genes (DEGs) (Table 1). Among these, eight genes were induced in the overexpression line, including OsbHLH044, and 31 genes were repressed, suggesting that OsbHLH044 mostly acts as a repressor. DEGs were annotated and categorized according to their putative or demonstrated functions based on gene ontology annotation and published data (Table 1). Six out of 39 DEGs were previously found to be misregulated after ectopic CRL1 expression induction in the crl1 mutant background (Lavarenne et al., 2019) (Table S2). Five of them were coherently down- or upregulated in both data sets. Altogether, this suggests that OsbHLH044 negatively regulates a gene regulatory subnetwork downstream of CRL1. According to the function of the identified DEGs (Table 1), this subnetwork could be related to the control of programmed cell death and senescence processes that could be involved later in root development in the differentiation of aerenchyma.

DISCUSSION

In this study, we explored how CRL1 regulates crown root formation in rice. We identified a new CRL1 DNA binding motif that we called the CRL1-box (CACA(A/C)C). Both CRL1 and AS2 can bind the previously described LBD-box (GGGCGC) and the newly identified CRL1-box in vitro and in vivo. The LBD-box was first determined as an LBD transcription factor DNA binding site with AS2, the founder member of the LBD family (Husbands et al., 2007), and is bound by several other LBD transcription factors, such as RTCS in maize (Majer et al., 2012; Muthreikh et al., 2013; Xu et al., 2015), HvRAMOSA2 in barley (Hordeum vulgare) (Koppolu et al., 2013), and AtLBD18 in Arabidopsis (Bell et al., 2012; Berckmans et al., 2011). LBD transcription factors bind DNA as dimers and can recognize pairs of LBD-boxes with different affinities depending on the number of spacing bases (Chen et al., 2019). It was shown that AtLBD18 regulates the expression of AtEXP14 by binding to its promoter. However, the DNA binding region of AtLBD18 in the AtEXP14 promoter does not contain an LBD-box (Lee et al., 2013), suggesting that LBD transcription factors can bind different DNA sequences. This was also observed for AtLBD15, involved in tracheary element differentiation in Arabidopsis roots, which binds a consensus DNA sequence (CATTAT) that is different from the LBD-box (Ohashi-Ito et al., 2018). Altogether, this suggests that LBD transcription factors have a relaxed DNA binding specificity, which could explain why they are involved in the regulation of different developmental programs. Relaxed DNA binding specificity is also observed for other plant transcription factors. For example, the MYELOBLASTOSIS (MYB) superfamily Ph3 transcription factor from petunia (Petunia hybrida Vilm.) binds two distinct DNA sequences, MYB binding sequence 1 (MBS1) (TCA(A/C)C) and MBS2 (AGT-CATGGTGT) (Solano et al., 1997). Similarly, plant R2R3-MYB proteins, which regulate a myriad of processes, can bind several distinct DNA binding sequences (Kelemen et al., 2015; Proute & Campbell, 2012).

Heterodimerization or interaction with other proteins can be required for DNA binding or can modify the affinity for the binding site of LBD transcription factors. For instance, it was shown in Arabidopsis that LD818/LDB3 heterodimerization is required to induce the expression of E2Fa, a gene involved in asymmetric cell division during lateral root initiation (Berckmans et al., 2011). In maize, RTCS and RTCL, encoded by two LBD paralogs that are involved in crown root formation, can bind the LBD-box as homo- and heterodimer (Majer et al., 2012; Xu et al., 2015). The bHLH048 transcription factor regulates the properties of AS2 by reducing the affinity of AS2 for the LBD-box (Husbands et al., 2007). In this context, it would be interesting to know which protein could interact with CRL1 and how these interactions could modulate the specificity or affinity of CRL1 with DNA sequences. This could be a key to better understand CRL1-mediated regulation of genes involved in crown root initiation.

Here, we demonstrated also that in vivo, CRL1 can trans-activate genes whose promoter contains at least one LBD-box or CRL1-box, but not those devoid of these sequences in their promoters. This suggested that these former genes are CRL1 primary targets and that they can contribute downstream of CRL1 in crown root formation. For instance, QHB is known to be involved in QC differentiation and maintenance, and specifically expressed in quiescent center cells of rice seminal root and crown root (Kamiya et al., 2003; Ni et al., 2014). QHB expression was previously identified to be positively regulated by CRL1 and downregulated in the crl1 mutant (Coudert et al., 2011; Coudert et al., 2015; Inukai et al., 2005; Lavarenne et al., 2019; Liu et al., 2005). Recently, QHB was suggested to be regulated by CRL1 via a regulatory cascade including other elements such as auxin signaling components and genes controlling crown root initiation (Lavarenne et al., 2019). Thus, the
### Table 1 OsbHLH044 acts as a repressor of transcription

| RAP-DB ID   | MSU ID            | Fold Change | P-value      | Annotation                                                                 | Function in rice                                                                 | Expression in rice                  | References                        |
|-------------|-------------------|-------------|--------------|----------------------------------------------------------------------------|--------------------------------------------------------------------------------|-------------------------------------|-----------------------------------|
| Os03g0188400| LOC_Os03g08930    | 10.15       | 2.76E-16     | OsbHLH044, basic Helix–loop–helix DNA-binding protein 44                   | Anthers development                                                              | Roots, seeds and panicles          | Sato et al., 2013 and Yang et al., 2016 |
| Os04g0552000| LOC_Os04g46630    | 1.91        | 0.0015       | OsEXPB15, beta-expansin family                                            | ND                                                                              | ND                                 | ND                                |
| Os11g0434800| ND                | 1.76        | 0.0003       | Hypothetical protein                                                        | ND                                                                              | ND                                 | ND                                |
| Os08g0387400| LOC_Os08g29770    | 1.64        | 0.0017       | OsGH9B13, Glycoside hydrolase                                               | ND                                                                              | ND                                 | ND                                |
| ND          | LOC_Os08g20350    | 1.59        | 0.0006       | Retrotansposon                                                              | ND                                                                              | ND                                 | ND                                |
| Os06g0468300| ND                | 1.59        | 0.0005       | Hypothetical protein                                                        | ND                                                                              | ND                                 | ND                                |
| Os04g0172100| ND                | 1.53        | 0.0031       | Hypothetical protein                                                        | ND                                                                              | ND                                 | ND                                |
| Os09g0532400| LOC_Os09g36220    | 1.51        | 0.0012       | OsPRR95, Pseudo-response regulator                                          | Circadian-associated rice pseudo response regulator, control of flowering time. | Leaves. May be ABA- and ET-responsive (motif on the promoter) | Murakami et al., 2005             |
| Os01g0910500| ND                | −1.51       | 0.00000431   | Hypothetical protein                                                        | Role in NO-mediated leaf cell death.                                              | ND                                 | ND                                |
| Os03g0131200| LOC_Os03g03910    | −1.51       | 0.0015       | OsCatC, Catalase C, Hydrogen peroxide-induced leaf cell death                | ND                                                                              | Blades, panicles, leaf sheaths, and culms, but lower in roots                 | Lin et al., 2012                  |
| Os01g0148900| LOC_Os01g05650    | −1.54       | 0.0001       | OsMT2a, Metallothionein-like protein type 2                                  | High content of cysteine residues that bind various heavy metals.                | Stems, leaves, rachis, inflorescences and seeds. Induced by sucrose starvation, heat shock, ABA, salt, and Cd. Downregulated by Zn and \( \text{H}_2\text{O}_2 \). | Kim & Kang 2018                   |
| Os06g0552900| LOC_Os06g35940    | −1.54       | 0.0024       | OsFTL12, FLOWERING TIME-LIKE GENE 12, similar to SP3D                      | ND                                                                              | Phloem sap                         | Aki et al., 2008                  |
| Os01g0975300| LOC_Os01g74410    | −1.57       | 0.0000223    | OsMYB48, MYB family transcription factor                                   | Drought and salinity tolerance by regulating stress-induced ABA synthesis genes  | Stems, sheaths, leaves and panicles, but mainly expressed in roots            | Xiong et al., 2014                |
| Os12g0222300| LOC_Os12g12090    | −1.58       | 0.0000764    | Hypothetical protein                                                        | ND                                                                              | ND                                 | ND                                |
| Os02g0765600| LOC_Os02g25710    | −1.59       | 0.0002       | Hypothetical protein AMY1A, Alpha-amylace and starch granule degradation     | Degradation of starch granules. GA-responsive                                    | Expressed in all tissues. Downregulated by ABA and GA-responsive.              | Huang et al., 1990; Hwang et al., 1999; Kitajima et al., 2009 and Zhang et al., 2014 |
| ND          | LOC_Os08g15990    | −1.61       | 0.0007       | 500 bp downstream of retrotransposon                                        | ND                                                                              | ND                                 | ND                                |

(continued)
| RAP-DB ID       | MSU ID            | Fold Change | P-value | Annotation                                                                 | Function in rice                                      | Expression in rice          | References                                      |
|-----------------|-------------------|-------------|---------|-----------------------------------------------------------------------------|-------------------------------------------------------|-----------------------------|-------------------------------------------------|
| Os03g0276500    | LOC_Os03g16860    | -1.61       | 0.0065  | OsHsp71.1, Similar to Heat shock protein 70                                | Involved in anther and seed development. Plays a role in abiotic stress response. | Induced by salt and ABA     | Zou et al., 2009; Oono et al., 2010 and Fu et al., 2017 |
| Os08g0529800    | LOC_Os08g41780    | -1.63       | 0.0001  | Alpha/beta hydrolase fold-1 domain containing protein. Triacylglycerol lipase precursor | ND                                                   | ND                           | ND                                              |
| Os07g0683900    | LOC_Os07g48490    | -1.64       | 0.000000346 | Ricin B-related lectin domain containing protein                           | ND                                                   | ND                           | ND                                              |
| Os04g0119800    | LOC_Os04g02920    | -1.64       | 0.0014  | Leucine-rich repeat domain containing protein                              | ND                                                   | Expresed in leaves, roots, and embryos            | Sato et al., 2013 |
| Os03g0405500    | LOC_Os03g29190    | -1.64       | 0.0000657 | Similar to protein disulfide isomerases-like protein                       | ND                                                   | ND                           | ND                                              |
| Os08g0131200    | LOC_Os08g03690.1  | -1.71       | 0.0000712 | OsLTP1.7, Similar to Non-specific lipid-transfer protein                     | ND                                                   | ND                           | ND                                              |
| Os11g0971000    | LOC_Os11g73940.1  | -1.71       | 0.0003  | Hypothetical protein                                                        | ND                                                   | ND                           | ND                                              |
| Os01g0971000    | LOC_Os12g31120    | -1.78       | 0.0071  | ND                                                                           | ND                                                   | ND                           | ND                                              |
| Os11g0255300    | LOC_Os11g14900    | -1.8        | 0.00063 | OsCP1, Cysteine protease 1                                                  | Involved in programmed cell death and anther and pollen development | Under the control of a bHLH gene                   | Zhang et al., 2011 and Ji et al., 2013 |
| Os10g0100700    | LOC_Os10g01080    | -1.8        | 0.0035  | OsPDX1.3b, Pyridoxine biosynthesis protein 1.3b                            | ND                                                   | ND                           | ND                                              |
| Os10g0552600    | LOC_Os10g40510.1  | -1.95       | 0.0009  | OsHyPRP18, hybrid proline-or glycine-rich protein 18                       | ND                                                   | ND                           | ND                                              |
| Os01g0907600    | LOC_Os01g67980    | -2.03       | 0.0002  | OsSAG12-1, senescence associated gene 12–1                                 | Involved in programmed cell death and digestion of storage proteins | Induced by GA and ABA                  | Shintani et al., 1997; Kato et al., 2003; Singh et al., 2013; Diaz-Mendoza et al., 2016 and Uji et al., 2017 |
| Os01g0810300    | LOC_Os01g59530    | -2.11       | 0.000088 | OsCaM61, Calmodulin 61                                                     | Signal transducer in abiotic stress response         | All tissues                   | Chinpongpanich et al., 2011 and 2012; Tayade et al., 2018 |
| Os12g0516200    | LOC_Os12g33150    | -2.18       | 0.0008  | Hypothetical protein                                                        | ND                                                   | ND                           | Sheu et al., 1996; Hwang et al., 1999; Hakata et al., 2012 and Liu et al., 2019 |
| Os09g0457800    | LOC_Os09g28420    | -2.29       | 0.0003  | Hypothetical protein                                                        | Degradation of starch                                | ND                           | ND                                              |

Table 1. (continued)
| RAP-DB ID    | MSU ID        | Fold Change | P-value       | Annotation                                                                 | Function in rice                                                                 | Expression in rice | References                                                                 |
|-------------|---------------|-------------|---------------|-----------------------------------------------------------------------------|-----------------------------------------------------------------------------------|-------------------|---------------------------------------------------------------------------|
| Os07g0529000 | LOC_Os07g34520| −2.49       | 0.000000315   | OsEnS-107, isocitrate lyase, endosperm-specific gene 107                    | Involved in drought tolerance, defense response, storage lipid mobilization, and leaf senescence. | All tissues       | Lee et al., 2001; Liang et al., 2014; Ramegowda et al., 2014; Wu et al., 2016; Mao et al., 2017; Sun et al., 2017 |
| Os01g0975900 | LOC_Os01g74450| −2.58       | 0.00000166    | OsTIP1.2, Tonoplast intrinsic protein 1-2                                   | Water transporter involved in abiotic stress resistance.                           | Upregulated by PEG, salt, and ABA and downregulated by chilling. More highly expressed in roots after drought and salt stress. | Li et al., 2008; Zhang et al., 2012, Nguyen et al., 2013                  |
| Os03g0103100 | LOC_Os03g01300| −2.85       | 0.0036        | OsHyPRP03, hybrid proline- or glycine-rich protein 3                         | Involved in abiotic and biotic stress resistance                                  | Expressed in all tissues, upregulated by abiotic and biotic stress                | Chen et al., 2015; Silveira et al., 2015; Byun et al., 2018              |
| Os01g0200700 | LOC_Os01g10400| −3.41       | 0.0000169     | OsMT3a, Metallothionein-like protein type 3                                  | Plays a role in metal homeostasis and ROS scavenging.                            | Upregulated by abiotic stress                                                   | Yamauchi et al., 2017                                                   |
| Os07g0684000 | LOC_Os07g48500| −3.54       | 0.000000175   | OrysaEULS2, Euonymus europaeus lectin domain 2                               | Role in sensing and responding to multiple environmental cues. Carbohydrates binding | Expressed in shoot and root. Induced by ABA, JA, salt, and pathogens.           | Moons et al., 1997; Atalah et al., 2012 and 2014; Schutter et al., 2017 |
| Os08g0250000 | ND            | −6.32       | 1.24E-09      | OsMT3, Metallothionein-like protein 3                                        | Plays a role in metal homeostasis and ROS scavenging.                            | Upregulated by abiotic stress                                                   | Yamauchi et al., 2017                                                   |
| ND          | LOC_Os02g54090| −7.47       | 0.0007        | Hypothetical protein                                                         | ND                                                                                | ND                            | Zhou et al., 2006; Yamauchi et al., 2017                                  |
| ND          | LOC_Os05g11320| −32.15      | 1E-10         | OsMT3b, Metallothionein-like protein 3B                                       | Plays a role in metal homeostasis and ROS scavenging.                            | ND                            |                                                                           |

Differentially expressed genes between OsbHLH044_crl1OE and N_OsbHLH044_crl1OE lines with fold change $\geq 1.5$ and $P < 0.01$. ND, not determined.
identification of a CRL1-box in its promoter and its transactivation in rice protoplast assays suggest that QHB is a direct target of CRL1. The promoter of RICE OUTMOST CELL-SPECIFIC4 (ROC4) also contains a CRL1-box. Its expression co-localizes with CRL1 expression during crown root primordia formation (Coudert et al., 2015). Our data show that it is transactivated by CRL1 in transient protoplast assays. ROC4 is also expressed during lateral root development in rice and in the epidermis of the crown root apex but not in the mature root (Ito et al., 2003; Takehisa et al., 2012; Wei et al., 2016). Moreover, it was shown that ROC4 is involved in the regulation of genes involved in cell wall precursor metabolism and by consequence in the specification of outer cell layers such as epidermis, exodermis, and sclerenchyma (Huang et al., 2012; Ito et al., 2003). Its closest homologs in Arabidopsis, HOMEO.DOMAIN GLABROUS1 (HDG1, At3g61150) and ANTHOCYANIN-LESS2 (ANL2, At4g00730), are involved in lateral root development and in meristem cell proliferation and root radial patterning (Horstman et al., 2015; Kubo et al., 1999; Kubo & Hayashi, 2011; Mabuchi et al., 2016; Nakamura et al., 2006). Altogether, the data suggest that ROC4 is likely involved in crown root primordia formation downstream of CRL1.

The binding capacity of CRL1 in planta was assessed by ChIP-qPCR assay using the DXCH lines which express a CRL1-HA fusion protein under the control of a DEX-inducible system in a rice crl1 mutant background. The promoters of two CRL1 target genes, OsROP and OsbHLH044, genes, which contain both CRL1- and LBD-boxes, were analyzed. Data showed that for both promoters, CRL1 did not bind the promoter region that contains the CRL1-box even if it also contains an LBD-box but binds the promoter region that contains only LBD-boxes. This showed that in the context of these two promoters, CRL1 preferentially binds certain LBD-boxes. This suggests that these cis-regulatory elements do not have the same accessibility for CRL1. The accessibility of cis-regulatory elements for a transcription factor in planta can be modulated by chromatin structure, DNA methylation, or the interaction with another protein in its near vicinity (Schmitz et al., 2022). Nevertheless, our data revealed that OsROP and OsbHLH044 constitute direct CRL1 target genes in rice stem bases.

We further analyzed the biological function of these two genes using knockout mutants generated by CRISPR/Cas9-mediated gene editing and using overexpression in different genetic backgrounds. Several independent allele-specific knockout lines in those genes have a reduced crown root number compared to WT lines. Overexpression of these genes in the WT Taichung 65 background enhances the crown root number. Their effect on crown root development is observable at the early plant development stage before tillering, which suggests that they primarily act on crown root development and that the modification of the crown root number can influence later tiller development. However, their overexpression in the crl1 mutant background was not sufficient to rescue crown root development, suggesting that other CRL1-regulated genes are necessary for the formation of crown roots. Altogether these data demonstrated that OsROP and OsbHLH044 act directly downstream of CRL1 in the gene regulatory network and contribute to crown root formation.

OsROP encodes a Rho-related GTPase from plants (ROP) family protein that controls the actin cytoskeleton structure and cell polarity establishment through the modulation of calcium or auxin signaling pathways (Nibau et al., 2006). Its expression pattern co-localizes with CRL1 mRNA expression in crown root primordia (Coudert et al., 2015). The expression of OsROP is upregulated in response to microgravity in rice callus but its function needs to be studied further (Jin et al., 2015). Its homolog in Arabidopsis, AT4G35750, has been associated with membrane trafficking, cytoskeleton dynamics, and the lipid signaling-mediated plant response to pathogens (Ajambang et al., 2016). In Arabidopsis, several proteins belonging to the ROP family are associated with root development (Feiguelman et al., 2018). ROP4 and ROP6 are expressed in root meristems and the root epidermides, where they contribute to root hair elongation. Their role in the dynamics and organization of actin is suggested by the fact that they co-localize with a myosin and actin-enriched zone, corresponding to the actin-organizing centers during mitosis and cell elongation (Molendijk et al., 2001). Furthermore, downstream of the auxin signaling pathway, ROP6 is also involved in the subcellular distribution of PIN1 and PIN2 during the root gravitropic response (Han et al., 2018; Lin et al., 2012).

OsbHLH044 encodes a bHLH transcription factor and its expression is induced by cytokinins in rice crown roots (Sato et al., 2013). In Medicago truncatula, the OsbHLH044 homolog MtbHLH1 is specifically expressed in the root meristematic zone, dividing root pericycle cells, lateral root primordia, and cortical cells of the main root at the site of lateral root emergence, suggesting that it could play a role in both lateral root formation and emergence (Godiard et al., 2011). This transcription factor could play a conserved role in lateral and adventitious root formation in angiosperms. We found that many genes were downregulated by OsbHLH044, suggesting that it acts as a transcription repressor. Other plant bHLH transcription factors act as negative regulators (Huq & Quail, 2002; Oh et al., 2004; Tian et al., 2015). This is for example the case for bHLH129 in Arabidopsis, which represses ABA signaling genes and promotes root elongation when overexpressed in Arabidopsis (Tian et al., 2015). Here we showed that many genes repressed by OsbHLH044 overexpression have a function related to the stress response. For instance, in OsbHLH044_crl1OE lines, the expression of CATALASE C...
et al., 2008; Nguyen et al., 2016; Silveira et al., 2015; Van Holle & Van Damme, 2018; Xiong et al., 2014; Zhang et al.,

CRL1-mediated OsbHLH044 expression is transiently upregulated after DEX-AC233899.1 roots by activating key regulatory genes such as

Madison, WI, USA) as described before (Coudert et al., 2015).

2005) was used for gene overexpression and

nesis using CRISPR/Cas9. For seed production, plants were grown

that contribute to the process. In particular, we

showed that during the early steps of crown root forma-

tion, CRL1 controls a set of genes involved in programmed

cell death and senescence

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EXPEDERIMENTAL PROCEDURES

Plant material

The O. sativa L. cv. Taichung 65 crl1 mutant line (Inukai et al., 2005) was used for gene overexpression and O. sativa cv. Kitaake was used for protoplast production and for gene-targeted mutagenesis using CRISPR/Cas9. For seed production, plants were grown under a 14/10 h light/dark photoperiod at 28°C (day)/25°C (night) with a relative humidity of 80% (day)/70% (night) in 3-L pots (Soparco 7019, Soparco Condé-sur-Huisne, France) filled with Jiffy professional substrate (Jiffy eco140/GOM2, Jiffy, Trevoux, France) and supplemented every 2 weeks with chemical fertilizer during vegetative and reproductive growth (N/P/K 20:12:1 and 8:16:3, respectively; Dynaflo, Frontignan, France). Seeds were hull, disinfected by incubation for 3 min in ethanol 70% (v/v) and then for 90 min in 3.8% (v/v) sodium hypochlorite solution containing 1% (v/v) Tween-80, and finally rinsed four times with milli-Q water.

CRL1 protein production in E. coli and purification

Full-length CRL1 cDNA was amplified by reverse transcription-PCR (RT-PCR) and cloned into pGEM-T (Promega A3600, Promega, Madison, WI, USA) as described before (Coudert et al., 2015).
probe of the following round of SELEX. After 10 cycles of SELEX, the obtained DNA products amplified by PCR were cloned into the TOPO-TA vector (Invitrogen 450071, Invitrogen, Carlsbad, CA, USA). In total, 16 and 43 independent bacterial clones for MBP-4ZF and MBP-CRL1, respectively, were sequenced and aligned with the MEME program (http://meme.sdsc.edu/meme4_1/cgi-bin/meme.cgi) to determine the conserved DNA sequences.

cDNA cloning for transient and stable transformations

A CRL1-GFP fusion protein was obtained by amplifying the cDNA cloning for transient and stable transformations with the MEME program (http://meme.sdsc.edu/meme4_1/cgi-bin/USA). In total, 16 and 43 independent bacterial clones for MBP-the obtained DNA products amplified by PCR were cloned into the cohesive ends for further cloning (Table S4). The corresponding thetized by GenScript (Piscataway, NJ, USA) and annealed to CRL1-box cDNA sequence without stop codon from a plasmid (Coudert et al., 2003): Two cDNA sequences corresponding to putative targets of CRL1 were obtained from the cDNA-KOME database (Kikuchi et al., 2003): OsROP and OsbHLH044 are direct targets of CRL1 and OsbHLH044, which was inserted by ligation into the binary vector pCAMBIA5300, upstream of a minimal pVS1 promoter and the GUS-encoding gene. For all constructs, successful cloning was verified by sequencing, and mRNA expression was measured using Centrimo from the MEME suite (Bailey & MacHank, 2012).

**Protoplast transient transformation, reporter enzyme assays, and fluorescence microscopy**

Protoplasts obtained from leaves and shoots of 9-day-old etiolated rice seedlings were transiently transformed using the protocol of Cacas et al. (2017) with few modifications related to rice protoplast isolation. After dissection, hulled rice cv. Kitaake seeds were incubated under dark sterile conditions in pots containing half-strength Murashige and Skoog (MS) medium (Duchefa M0231, including Gamborg B5 vitamin, Duchefa Biochemie B.V., Haarlem, the Netherlands) with 3.5 g per liter of plant agar (Duchefa P1001, Duchefa Biochemie B.V., Haarlem, the Netherlands). After slicing the rice seedlings into small pieces, vacuum was applied for 10 min to the rice seedling sections soaked in 30 ml of fungal enzymatic solution (1.5% [w/v] cellulase R10 [Duchefa C8001, Duchefa Biochemie B.V., Haarlem, the Netherlands]) and 0.4% [w/v] macerozyne R10 [Duchefa M002, Duchefa Biochemie B.V., Haarlem, the Netherlands]), followed by incubation in dark conditions for 4 h at 26°C in order to remove cell walls. Rice protoplasts were co-transformed with (i) pGusSH-47 reporter plasmids carrying the GUS reporter gene under the control of (1) a minimal CaMV 35S -47 promoter and the GUS-encoding gene, or (ii) pGusSH-47 reporter plasmids carrying the GUS reporter gene under the control of (1) a minimal CaMV 35S -47 promoter and the GUS-encoding gene, or (iii) the empty pRT101 effector plasmid or the pRT101 plasmid carrying CRL1 or AS2 cDNA under the control of a CaMV 35S 47 promoter. Protoplasts were transformed using polyethylene glycol as described previously (Yoo et al., 2007) with the three types of plasmids in a reporter: normalization:effector plasmid ratio of 2:2:6. The protoplasts were collected 18 h after transformation and frozen in liquid nitrogen. GUS and LUC activities were measured as described previously (Zarei et al., 2011) using a Fluoroskan Ascent (Labsystems/Thermo-Fisher, Waltham, MA, USA) and a Modulus Microplate (Turner Biosystems/Promega, Madison, WI, USA), respectively. LUC activity was used to correct for the differences in GUS activity that may be linked with variations in efficiency of transformation and protein extraction.

**Oryza Sativa cv. Taichung 65 genomic DNA was used as a template for the amplification of 1000-bp promoter fragments of OsROP (LOC_Os04g47330), OsHOX17 (LOC_Os04g46350), ROC4 (LOC_Os04g48070), and OsbHLH044 (LOC_Os03g08930, OsROP, proSHOX17, pROC4, and pOsbHLH044, respectively) or the 1500-bp promoter fragment of OsHB (LOC_Os1g63510, pOHB) using specific primers with enzymatic restriction sites (Table S3). The 1000-bp OsHOX14 promoter fragment (pOsHOX14) was synthesized by GenScript (GenScript, Piscataway, NJ, USA) using DNA sequences retrieved from RAP-DB (Os070581700) (Sakai et al., 2013). All PCR products were first cloned into the pGEM-T Easy vector (Promega A1360, Promega, Madison, WI, USA) according to the manufacturer’s instructions. The promoters were excised using enzymatic digestion (Table S3) and purified after agarose gel electrophoresis using the QIAquick Gel Extraction Kit (Qiagen 28704, Qiagen, Venlo, the Netherlands) according to the manufacturer’s instructions. Purification products were then inserted by ligation into the pGusSH-47 plasmid upstream of a minimal CaMV 35S -47 promoter and the GUS-encoding gene, except for pOsbHLH044, which was inserted by ligation into the binary vector pCAMBIA5300, upstream of a minimal pVS1 promoter and the GUS-encoding gene. For all constructs, successful cloning was verified by sequencing. Surveys for the cis-regulatory motifs in all promoters were performed using Centrimo from the MEME suite (Bailey & MacHank, 2012).
CRL1-GFP fluorescence localization was observed with an Axio Imager Z2 microscope (Zeiss, Marly-le-Roi, France) coupled to an X-cite 120 LED/LED illumination system (EXCELITAS Technologies, Waltham, MA, USA) and an Axio Cam 506 color camera (Zeiss, Marly-le-Roi, France). GFP was excited at 457–487 nm and emission was detected at 502–536 nm.

**Obtention of transgenic plants**

For gene-targeted mutagenesis via CRISPR/Cas9, two specific single-guide RNAs (sgRNAs) were designed per gene using the benchling CRISPR design tool (http://benchling.com) for OsROP and OsbHLH044 and integrated using LR clonase into the Agrobacterium tumefaciens strain EHA105 (Khong et al., 2015). Taichung 65 and the crtl mutant in the Taichung 65 background were used for gene overexpression and Kitaake was used for CRISPR/Cas9-mediated mutagenesis; both cultivars were genetically transgenic T2 line (without T-DNA) was kept as control (null sister lines). For overexpression in the Taichung 65 background of the CRL1-GFP fusion protein in response to DEX treatment in a bHLH044 mutant background, a transgenic OsbHLH044 mutant background was developed. The HA epitope coding sequence (TACCCATACGACGTCCCAGACTACGCT) was cloned in phase with the CRL1 cDNA was cloned between the I/XhoI restriction sites of the pINDEX2-CRL1-HA plasmid. Plants were transformed and homozygous monolocus plants were selected as described in (Coudert et al., 2003). For plants transformed for gene overexpression, monolocus and homozygous T-DNA lines were selected based on hygromycin resistance in the T1 and T2 generations (Os_crl1OE lines) (Khong et al., 2015). For overexpression in the crtl mutant background, a nontransgenic T2 line (without T-DNA) was kept as control (null sister N_Os_crl1OE lines). For osrop cas and osbhlh044 cas plants transformed for CRISPR/Cas9-mediated mutagenesis, T0 were genotyped by PCR using primers flanking the two sgRNA sequences (Table S3) and sequenced to select mutated lines. Homozygous mutated T1 lines without the T-DNA carrying the Cas9 sequence were selected by PCR analysis (for primers see Table S3) and sequenced. Then T2 lines without Cas9 were used for root phenotyping.

To perform ChiP-qPCR assays a transgenic O. sativa L. line, allowing the induction of the expression of a CRL1-HA fusion protein in response to DEX treatment in a crtl mutant background was developed. The HA epitope coding sequence (TACCCATACGACGTCCCAGACTACGCT) was cloned in phase with the CRL1 open reading frame just before the stop codon. The CRL1-HA cDNA was cloned between the Spel/Xhol restriction sites of the binary vector pInDEx2 (Ouwerkerk et al., 2001) to generate the pInDEx2-CRL1-HA plasmid. Plants were transformed and homozygous monolocus plants were selected as described in (Coudert et al., 2015). The obtained line used in this work was named DXCH.

**Root phenotyping**

For root system phenotyping of transgenic lines (osrop cas, osbhhlh044 cas, OsROP_crl1OE, and OsbHLH044_crl1OE) at an early stage before tillering, disinfected hulled seeds were incubated in Petri dishes (ref. 82.1473.001, Starstedt, Nümbrecht, Germany) containing a filter pad (ref. 1001-090, Whatman paper, GE Healthcare, Buckinghamshire, UK) and 15 ml MS/2 medium including Gamborg B5 vitamin (Duchefa M0231, Duchefa Biochemie B.V., Haarlem, the Netherlands) and placed in culture chambers under a 14/10 h light/dark photoperiod at 27°C and at a relative humidity of 70%. After 3 days, the plantlets were transferred into glass tubes (ref. 26.131.36.09, Schott Duran, Wertheim, Germany) containing MS/2 medium (Duchefa M0231, including Gamborg B5 vitamin, Duchefa Biochemie B.V., Haarlem, the Netherlands) in culture chambers under a 14/10 h light/dark photoperiod at 27°C and at a relative humidity of 70%. For late vegetative stage phenotyping experiments, Os_TC65OE lines were grown in net houses of the Agricultural Genetics Institute of Hanover in summer 2020. Plants were grown using sand columns (15 cm diameter x 35 cm height) according to the protocol described by Phung et al. (2016). After 4 weeks, when plants began tillering, root samples were harvested by removing sand and washing the roots in tap water. The crown roots and tillers were counted. For osrop cas and osbhlh044 cas lines, plants were grown in 3-L pots filled with Jiffy GOM2 compost (Jiffy eco140/GOM2, Jiffy, Trevoux, France) mixed with Florallye Blue Latex fertilizer (10N8P-10 K) (125 g for a 70-L soil bag) in a greenhouse at IRD in France under a 12/12 h light/dark photoperiod, at a temperature of 28°C (day)/25°C (night), and at a relative humidity of 70% (day)/60% (night). Sowing was done with four seeds per pot, with thinning at one plant per pot 1 week after sowing. After 4 weeks the crown roots and tillers were counted.

**Tissue sampling, RNA extraction and preparation**

For transcriptomic analysis, selection of the transgenic offspring, and RT-qPCR, disinfected hulled seeds were sown in Petri dishes (ref. 82.1473.001, Starstedt, Nümbrecht, Germany) containing a wet filter pad (ref. 1001-090, Whatman paper, GE Healthcare, Buckinghamshire, UK) with 15 ml MS/2 medium including Gamborg B5 vitamin (Duchefa M0231, Duchefa Biochemie B.V., Haarlem, the Netherlands) and placed in culture chambers under a 14/10 h light/dark photoperiod at 27°C and at a relative humidity of 70%. After 5 days, the plantlets were transferred into 250-mL wide-collar Erlenmeyer flasks containing 30 ml MS/2 medium including Gamborg B5 vitamin (Duchefa M0231, Duchefa Biochemie B.V., Haarlem, the Netherlands) for 24 h. The experiment was repeated independently three times with pC3500.0E-OsROP or -OsbHLH044 overexpression (OsROP_crl1OE or OsbHLH044_crl1OE, respectively) lines using their null sister lines (N_Os_crl1OE or N_Os_crl1OE) as controls. For each biological replicate, 10 to 15 stem bases were collected and immediately frozen in liquid nitrogen. After grounding 8–12 stem bases in liquid nitrogen using a TissueLyser II tissue disruption system (Qiagen, Venlo, the Netherlands) with 3-mm steel beads for 15 sec at 30 Hz, RNA was extracted using the Plant RNAeasy Kit (Qiagen 74804, Qiagen, Venlo, the Netherlands) in the presence of DNeasy I (Qiagen 79254, Qiagen, Venlo, the Netherlands) using a purification column according to the manufacturer’s instructions. RNA was quantified at 260 nm with a NanoDrop-1000 spectrophotometer (ThermoFisher, Waltham, MA, USA).

**RT-qPCR**

cDNA synthesis was performed using 2 µg of total RNA extracted from rice stem bases as described in Section 4.9 and the
OsRop and OsbHLH044 are direct targets of CRL1

Omniscript RT reverse transcriptase kit (Qiagen 205111, Qiagen, Venlo, the Netherlands) with oligo(dT)15 primers (Promega C1101, Promega, Madison, WI, USA). The relative transcript abundance of selected genes was determined using an MX3005P system (Agilent, Santa Clara, CA, USA) and Brilliant III Ultra-Fast qPCR Master Mix (Agilent 600880, Agilent, Santa Clara, CA, USA) using ROX normalization dye. The range of primer efficiencies observed for all primer pairs used (Table S3) was between 1.9 and 2.1. Three technical replicates were performed for each cDNA replicate. In addition, melting curves were obtained for the reactions, revealing single-peak melting curves for all amplification products. The amplification data were analyzed using LinRegPCR software version 2016.2 (Ruijter et al., 2015), and the starting concentration of 5 μM to induce the expression of CRL1-HA. Three technical replicates were conducted for each ChIP sample. The value of ΔCt was calculated by normalizing the ChIPDNA Ct values to μcDNA Ct for each ChIP sample. The value of ΔCt was calculated by normalizing the ΔCt values of a DEX-treated sample (e.g., T3 and T9) to the ΔCt value of a non-DEX-treated sample (e.g., T0 control). Finally, the fold change was calculated using the ΔΔCt value.

Western blot assay for CRL1-HA detection

DXCH plantlets (pGOS::GVG::CRL1-HA) were prepared as described just above. After DEX induction, stem bases were harvested and ground in liquid nitrogen, powder from ground stem bases was added to the EpiQuick Plant ChIP kit (Epigentek, NY, USA) CP3C buffer, and the cells were broken using a KIMBLE Precast™ 1× Laemmli sample buffer (60 mM Tris-HCl, pH 7.4, 100 mM NaCl, 10 mM Na2EDTA–2H2O, 25 mM d-glucose, 5 mM EGTA, 5% [v/v] glycerol, 5 mM DTT). Subsequently, 10 μL of 5x Laemmli sample buffer (60 mM Tris-HCl, pH 8.8, 2% [v/v] SDS, 10% [v/v] glycerol, 5% [v/v] β-mercaptoethanol, 0.01% [v/v] bromophenol blue) was added to 40 μL of protein extraction buffer (20 mM Tris-HCl, pH 7.4, 100 mM NaCl, 10 mM Na2EDTA–2H2O, 25 mM d-glucose, 5 mM EGTA, 5% [v/v] glycerol, 5 mM DTT). Subsequently, 10 μL of 5x Laemmli sample buffer (60 mM Tris-HCl, pH 8.8, 2% [v/v] SDS, 10% [v/v] glycerol, 5% [v/v] β-mercaptoethanol, 0.01% [v/v] bromophenol blue) was added to 40 μL of protein extraction solution that was used for the Western blot assay. After SDS-PAGE using a 12% Mini-PROTEAN® TGX™ Precast Protein Gel (Bio-Rad, CA, USA), immunoblotting was realized using a monoclonal anti-HA-tag antibody (ThermoFisher Scientific, MA, USA) and StarBright™ Blue 520 Fluorescent Secondary
Antibody (Bio-Rad). The Western blot images were produced using a Typhoon™ FLA 7000 biomolecular imager (GE Healthcare, IL, USA).

**Statistical analysis**

Data were analyzed using the Student’s t-test in Microsoft Excel 2010 (Microsoft Corporation, Redmond, WA, USA).

**ACCESSION NUMBERS**

Accession numbers of the genes discussed are presented in Table 1 and Tables S1 and S2.

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**AUTHOR CONTRIBUTIONS**

PG, GTH, AC, JR, and CS directed the research; MG, YC, KJ, GTH, AC, and PG designed the experiments; MG, KJ, YC, JL, and GTH performed the statistical and mathematical analyses; MG and PG analyzed the literature on the genes discussed and studied; MG, YC, and KJ interpreted the data and wrote the article, with input from PG, LL, VB, HTG, AC, TB, and BP; MG realized the transient activation assays, conducted qPCR analysis, performed transcriptome experiments, and generated knockout plants with the help of HTN; GTH generated overexpression plants in crf1 and WT backgrounds; YC generated and KJ selected DXCH transgenic lines; MG, GTH, and MRNT designed the phenotyping experiments and phenotyped plants with the help of KJ, TVD, DM, SG, KB, and JRB; KJ realized the ChIP assay and performed ChIP-qPCR experiments; YC realized SELEX and gel shift experiments and scanned mutants with the help of MB; HTMT generated most constructs for transient activation and gene overexpression assays; all authors edited and agreed on the final article.

**DATA AVAILABILITY STATEMENT**

The data discussed in this publication have been deposited in NCBI’s Gene Expression Omnibus (Edgar, 2002) and are accessible through GEO Series accession number GSE147200.

**SUPPORTING INFORMATION**

Additional Supporting Information may be found in the online version of this article.

- Figure S1. MBP-CRL1 binds DNA in vitro.
- Figure S2. Identification of the 4ZF DNA binding sequence by SELEX.
- Figure S3. CRL1-GFP localizes into the nucleus.
- Figure S4. The LBD transcription factor family has relaxed DNA binding activity in rice protoplasts.
- Figure S5. Expression levels of CRL1 target genes at 3, 6, and 9 hours after CRL1 expression induction by dexamethasone in a crf1 mutant background.
- Figure S6. CRL1-HA protein is detected in nuclear extract and restores crown root formation after dexamethasone (DEX) treatment of DXCH rice lines.
- Figure S7. Expression levels of OsROP and OsbHLH044 in response to CRL1 induction.
- Figure S8. OsROP and OsbHLH044 CRISPR/Cas9 mutations.
- Figure S9. Effects of mutation and overexpression of OsROP and OsbHLH044 on tiller number.
- Figure S10. Crown root numbers in osrop cas and osbhlh044 cas mutant lines at an early stage of development before tillering.
- Figure S11. Overexpression of OsROP and OsbHLH044 in the crf1 mutant does not affect crown root development in rice.
- Table S1. Selected putative target genes of the CRL1 transcription factor.
- Table S2. Differential expression of OsbHLH044-responsive genes after CRL1 expression induction in the crf1 mutant background.
- Table S3. List of primers used.
- Table S4. Sequence of the cis-regulatory tetramers.

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