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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;

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Kong et al., 2017). ASL/LBD transcription factors are characterized by a conserved region of ca. 100 amino acids termed the ASYMMETRIC LEAVES2/LATERAL ORGAN BOUNDARIES (AS2/LOB) domain (Iwakawa et al., 2002; Shuai et al., 2002). In addition, two classes of ASL/LBD transcription factor are identified based on the presence (Class I) or absence (Class II) of a Leu-zipper-like coiled-coil motif into the AS2/LOB domain (Chanderbali et al., 2015; Coudert et al., 2013a; Coudert et al., 2013b; Shuai et al., 2002). Most of the Class II LBD proteins are involved in nitrogen metabolism regulation (Albinsky et al., 2010; Rubin et al., 2009), whereas Class I LBD proteins are involved in developmental processes, such as pollen development (Kim et al., 2015), leaf development (Husbands et al., 2007; Li et al., 2016), meristem sizing (Ma et al., 2017), and post-embryonic root initiation (Inukai et al., 2005; Liu et al., 2005; Okushima et al., 2007; Taramino et al., 2007).

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; Tromas 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 AS2/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; 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. Analysis of the 1-kb promoter region located upstream of the translation start codon of these 277 CRL1-regulated genes showed that approximately 42% of these promoters contain at least one *LBD-box* motif (GCGGCG), a sequence known to be recognized by several ASL/LBD transcription factors (Coudert et al., 2015; Husbands et al., 2007; Ma et al., 2017; Majer et al., 2012; Xu et al., 2015). This suggests that these genes might be direct targets of CRL1. However, this motif is not strongly enriched in the promoters of CRL1-regulated genes as compared to all rice gene promoters, where the LBD motif is retrieved in 36% of the promoters (Coudert et al., 2015). This suggests that CRL1 might regulate the expression of these genes indirectly *via* the involvement of other transcription factors or directly by interacting with another DNA binding sequence. Indeed, another binding motif (the CATTTAT sequence) was previously identified for the AtLBD15 transcription factor, while AtLBD18 was shown to be able to interact with promoter sequences devoid of an *LBD-box* (Lee et al., 2013; Ohashi-Ito et al., 2018).

In this study, using systematic evolution of ligands by exponential enrichment (SELEX), we identified a new DNA binding motif recognized by CRL1, which we named the *CRL1-box*. We demonstrated that CRL1 can bind both the *CRL1-box* and the *LBD-box in vitro* by gel shift experiments as well as *in vivo* using transient activation assays in rice. Block scanning mutagenesis in the *CRL1-box* revealed four nucleotides in the consensus motif that are highly important for the transcriptional activation by CRL1. We further showed that CRL1 can transactivate a subset of selected putative CRL1-regulated genes, whose promoter sequence contains at least one *CRL1-box* or an *LBD-box*. Chromatin immunoprecipitation–quantitative PCR (ChIP-qPCR) assays showed that *in planta* CRL1 binds preferentially the *LBD-box* in the promoters of two CRL1-regulated genes, *Rho GTPase* (*OsROP*) and *basic helix–loop–helix 44* (*OsbHLH044*), which contain both a *CRL1-box* and an *LBD-box*. CRISPR/Cas9-mediated mutation of these two CRL1 target genes showed that both genes promote crown root development. Finally, transcriptome analysis of *OsbHLH044*-overexpressing plants in a *crl1* mutant background suggested that this transcription factor acts as a transcriptional repressor of a gene regulatory module downstream of *CRL1* during crown root formation.

RESULTS

CRL1 binds a new DNA *cis*-regulatory sequence

In order to analyze its binding sites *in vitro*, the CRL1 protein fused to maltose binding protein (MBP) was produced in *Escherichia coli* and purified by affinity chromatography. The interaction between MBP-CRL1 and a radiolabeled degenerated DNA sequence (N18) was analyzed by electrophoretic mobility shift assay (EMSA) (Figure S1). MBP

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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

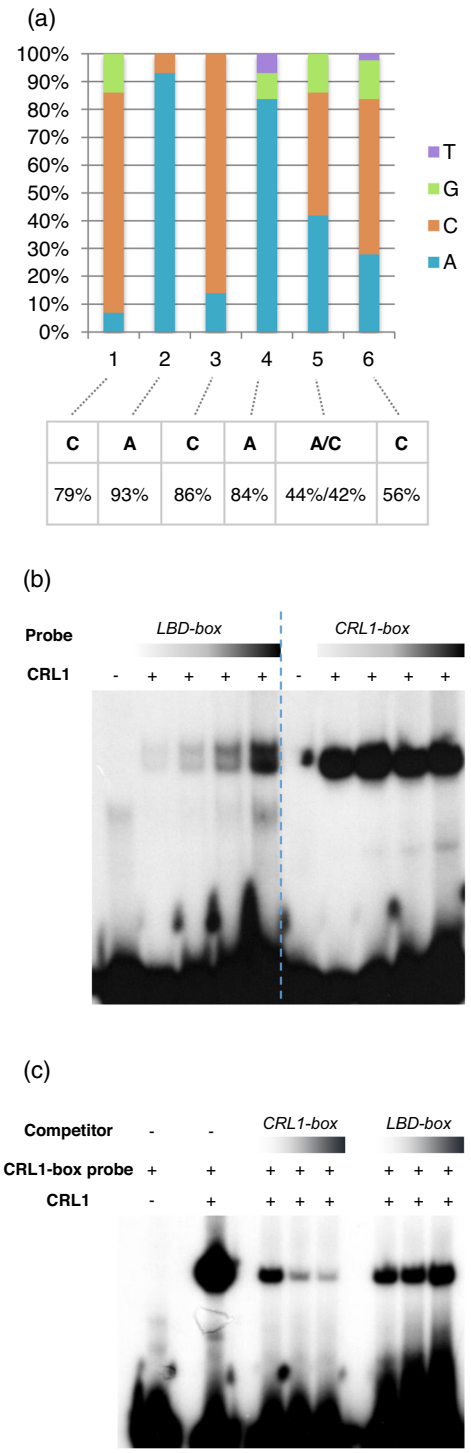

Figure 1. MBP-CRL1 binds both the *CRL1-box* and the LBD-*box in vitro*.
(a) Diagram showing the percentage of occurrence of each nucleotide on each of the six positions of the 43 sequences identified by SELEX. The lower part indicates the percentage of occurrence for each nucleotide defining the consensus sequence. (b) EMSA with MBP-CRL1 and increasing amounts of labeled *LBD-box* or *CRL1-box* probes (1, 2, 4, or 10 ng as indicated by the gray scale). (c) Competition EMSA assays using excess unlabeled *LBD-box* or *CRL1-box* (25, 100, or 250-fold as indicated by the gray scale) to interfere with the MBP-CRL1/*CRL1-box* interaction.

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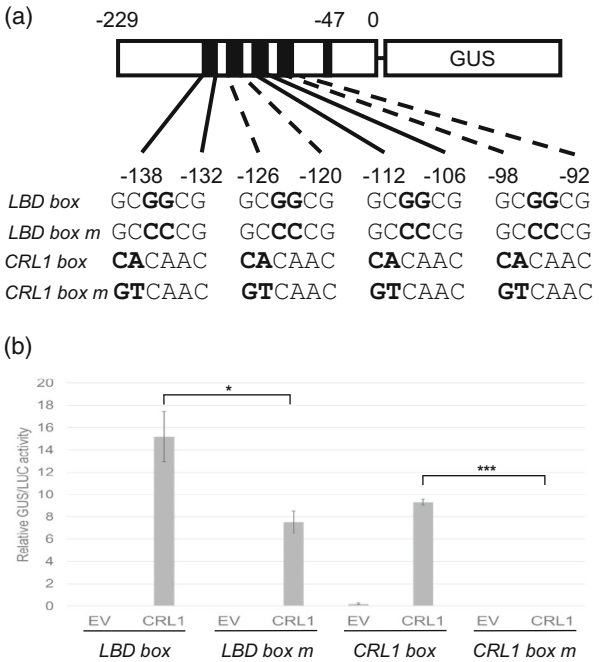

Figure 2. The *CRL1-box* and the *LBD-box* allow transactivation by CRL1 *in vivo*.
(a) Reporter constructs consisted of the GUS gene under the control of a minimal promoter (−47 to 0) and driven by enrichment of native or mutated (m) *cis*-regulatory sequences (−138 to −92). Bold nucleotides indicate 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 $P = 0.001$ (***). EV, empty vector.

(a)

Name	Code	DNA Sequence	Student's T-test with CRL1-box as reference
CRL1 box	CRL1 box	CACAAC	NS
CRL1 box mutated1	CM 1	**GT**CAAC	***
CRL1 box mutated 2	CM 2	C**TG**AAC	**
CRL1 box mutated 3	CM 3	CA**GT**AC	NS
CRL1 box mutated 4	CM 4	CAC**TT**C	**
CRL1 box mutated 5	CM 5	CACA**TG**	*
CRL1 box mutated 6	CM 6	**GTGTTG**	*

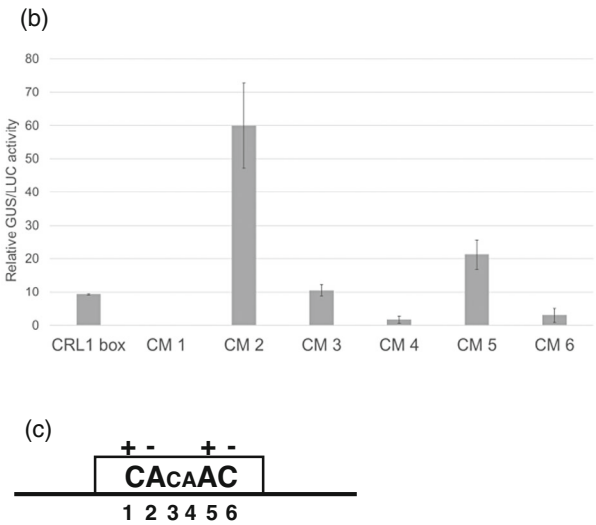

Figure 3. Block scanning mutagenesis of the *CRL1-box* using *in vivo* transactivation assays with CRL1 reveals most important bases in this sequence.
(a) Table of the different mutated DNA sequences of the *CRL1-box* used in the transient assay in rice protoplasts. Bold indicates nucleotide mutations.
(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 *CRL1-box* tetramers and its mutated form fused to GUS, (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 the 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 $P = 0.001$ (***). NS, no significance.
(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

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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

(a)

Gene	Annotation	*LBD-box*	*CRL1-box*
QHB	WOX	-	***CACACC***
OsbHLH044	bHLH	+	***CACACC***
OsHOX17	HD ZIP	-	-
OsROP	RhoGTPase	+	***CACACC***
ROC4	HD ZIP	-	***CACACC***
OsHOX14	HD ZIP	-	***CACAAC* and *CACACC***

(b)

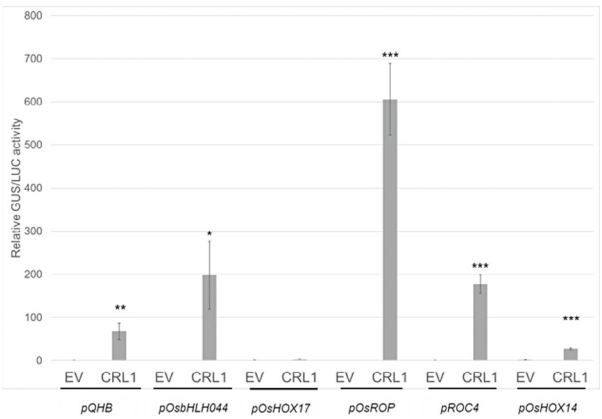

Figure 4. CRL1 is a transcriptional activator of the promoters of *QHB*, *OsbHLH044*, *OsROP*, *ROC4*, and *OsHOX14*.
(a) Selected putative target genes of CRL1 and survey of the *cis*-regulatory motif present in their promoters. (b) Transactivation of potential target gene promoters by CRL1 in rice protoplasts. Rice protoplasts were co-transformed with (i) reporter plasmids carrying the GUS reporter gene placed under the control of a minimal promoter and promoters of putative target genes of CRL1 (*pQHB*, *pOsbHLH044* *pOsHOX17*, *pOsROP*, *pROC4*, and *pOsHOX14*), (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 the 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 $P = 0.001$ (***). EV, empty vector.

ChIP-qPCR assays on the promoters of *OsROP* and *OsbHLH044*, which both contain *CRL1*- and *LBD-boxes*. For this purpose, a rice transgenic line expressing a CRL1-HA fusion protein (hereafter named DXCH) under the control of a dexamethasone (DEX)-inducible promoter in the *crl1* 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 S6a); 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 *crl1* 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

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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

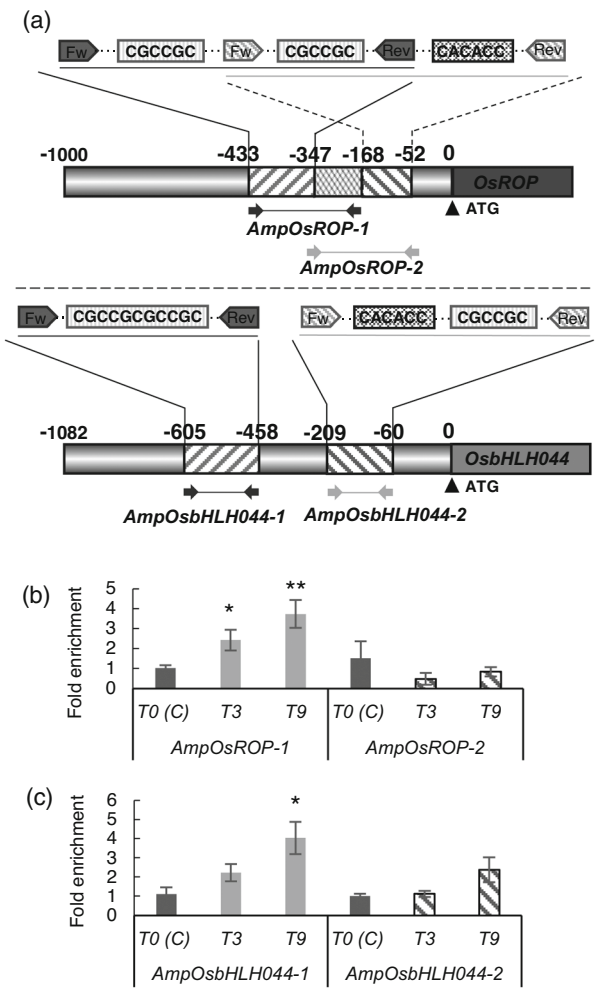

Figure 5. *In planta*, CRL1 binds preferentially to the *LBD-box* in the promoters of *OsROP* and *OsbHLH044*.
(a) Positions of primers relative to the transcription start site (arrows: Fw, forward primer; Rev, reverse primer) and of promoter regions amplified (*Amp*) after ChIP by qPCR in the promoters 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 $P = 0.001$ (***).

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 *OsbHLHO44* 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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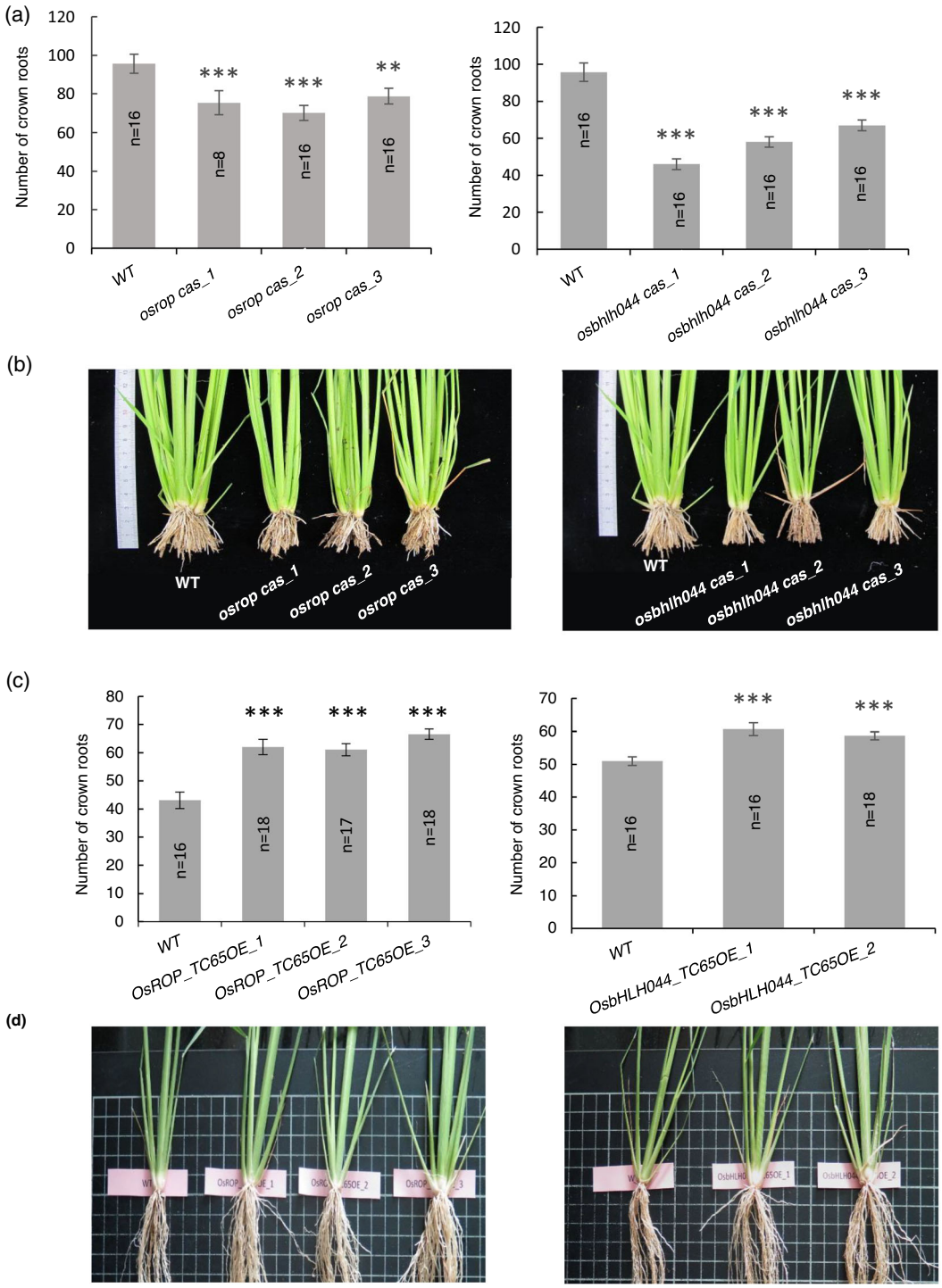

Figure 6. Mutation and overexpression of *OsROP* and *OsbHLH044* promotes crown root formation.
(a) Comparison of crown root number between WT and three independent *osrop cas* or three independent *osbhlh044 cas* lines. Student's *t*-tests were used to calculate the significant differences. Values were 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.

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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* (GCGGCG) 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; Muthreich 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 AtLDB18 regulates the expression of At*EXP14* by binding to its promoter. However, the DNA binding region of AtLBD18 in the At*EXP14* 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 (CATTTAT) 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 I* (*MBSI*) ((T/C)AAC(G/T)G(A/C/T) (A/C/T)) and *MBSII* (AGTTAGTTA) (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; Prouse & 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 LDB18/LBD33 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 transactivate 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

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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 panicules	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, Gycoside hydrolase	ND	ND	ND
ND	LOC_Os08g20350	1.59	0.0086	Retrotransposon	ND	ND	ND
Os06g0468300	ND	1.59	0.005	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	ND	ND	ND
Os03g0131200	LOC_Os03g03910	−1.51	0.0015	OsCatC, Catalase C, Hydrogen peroxide-induced leaf cell death	Role in NO-mediated leaf cell death.	Blades, panicles, leaf sheaths, and culms, but lower in roots	Lin et al., 2012
Os01g0149800	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 H_2O_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_Os02g52710	−1.59	0.0002	AMY1A, Alpha-amylase glycoprotein, degradation of starch granule	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)

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Table 1. (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 developement. 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	Expressed 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
Os01g0971000	LOC_Os01g73940.1	-1.71	0.0003	Hypothetical protein	ND	ND	ND
ND	LOC_Os12g31120	-1.78	0.0071	3 kb en aval de transposon protein, putative, CACTA, En/Spm subclass,	ND	ND	ND
Os11g0255300	LOC_Os11g14900	-1.8	0.000063	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	0.0000088	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	ND
Os09g0457800	LOC_Os09g28420	−2.29	0.0003	AMY3C, Alpha-amylase isozyme 3C precursor	Degradation of starch	Upregulated by GA and anoxic conditions, downregulated by ABA, salt, and sugar	Sheu et al., 1996; Hwang et al., 1999; Hakata et al., 2012 and Liu et al., 2018

(continued)

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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	ND
Os05g0202800	LOC_Os05g11320	−32.15	1E-10	OsMT3b, Metallothionein-like protein 3B	Plays a role in metal homeostasis and ROS scavenging.	Upregulated by abiotic stress. OsMT3b higher in rachises, leaves, and sheaths, but less in the roots, stems, and glumes	Zhou et al., 2006; Yamauchi et al., 2017

Differentially expressed genes between *OsbHLH044_crl1OE* and *N_OsbHLH044_crl1OE* lines with |fold change| > 1.5 and $P < 0.01$. ND, not determined.

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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, *HOMEODOMAIN GLABROUS1* (*HDG1*, *At3g61150*) and *ANTHOCYANINLESS2* (*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 C*RL1*- 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 allelic 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 calli but its function needs to be studied further (Jin et al., 2015). Its homolog in Arabidopsis, *AT4G35750*, has been associated with membrane trafficking signaling, 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 epidermis, 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*

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(*OsCATC*) (*LOC_Os03g0131200*), *CYSTEINE PROTEASE 1* (*OsCP1*) (*LOC_Os11g0255300*), *ENDOSPERM-SPECIFIC GENE 107* (*OsEnS-107*) (*LOC_Os07g0529000*), and *SENESCENCE ASSOCIATED GENE 12-1* (*OsSAG12-1*) (*LOC_Os01g0907600*), four genes involved in programmed cell death and senescence in rice, is repressed. *OsbHLH044* also represses three *METALLOTHIONEIN* genes (*OsMTs*) known to be involved in epidermal cell death and aerenchyma formation in rice (Jan et al., 2013; Ji et al., 2013; Lee et al., 2004; Singh et al., 2013; Yamauchi et al., 2017). *EUONYMUS EUROPAEUS LECTIN DOMAIN 2* (*OsEULS2*), *HYBRID PROLINE- OR GLYCINE-RICH PROTEIN 3* (*OsHyPRP03*), *CALMODULIN 61* (*OsCaM61*), *OsMYB48*, and *TONOPLAST INTRINSIC PROTEIN 1-2* (*OsTIP1*) are expressed in roots and involved in the stress response (Bouain et al., 2018; Li et al., 2008; Nguyen et al., 2016; Silveira et al., 2015; Van Holle & Van Damme, 2018; Xiong et al., 2014; Zhang et al., 2012). This is consistent with the downregulation of *AC233899.1*, the maize *OsbHLH044* homolog, in cortical cells of maize primary roots in response to ethylene treatment or in waterlogging conditions, two treatments that induce cell death and aerenchyma formation (Takahashi et al., 2015). *OsbHLH044* expression is transiently upregulated after DEX-mediated *CRL1* induction (Lavarenne et al., 2019).

Altogether these data show that CRL1 binds distinct DNA sequences and controls the development of crown roots by activating key regulatory genes such as *OsROP* or *OsbHLH044* that contribute to the process. In particular, we showed that during the early steps of crown root formation, CRL1 controls a set of genes involved in programmed cell death and senescence *via* OsbHLH044. The biological significance of this negative regulation during crown root formation will need further investigation.

EXPERIMENTAL 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 20:12:12 and 8:16:38, respectively; Dynaflor, 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). *CRL1*-specific primers containing restriction sites adapted for the cloning described hereafter were used (Table S3). The *CRL1* cDNA was ligated between the *Bam*HI/*Sal*I restriction sites of the pMal-c2x vector (New England Biolabs [NEB], Ipswich, MA, USA) in frame with the MBP gene to generate the pMal-c2x-CRL1 construct and express the MBP-CRL1 fusion protein. The C-terminal part of CRL1 was amplified and ligated between the *Nde*I/*Blp*I restrictions sites of pET14b to generate pET14b-CRL1ΔN108 and express the CRL1ΔN108-His protein (for primers see Table S3). CRL1ΔN108 was used to obtain anti-CRL1 antiserum. The pMal-c2x-4ZF plasmid containing the 4ZF coding sequence was obtained from Dr. S. Renault (Université François Rabelais, Tours, France).

The pMal-c2x-CRL1 or pMal-c2x-4ZF construct was transferred into *E. coli* strain BL21(DE3) (Promega L1195, Promega, Madison, WI, USA). Bacteria were cultivated in selective Luria Bertani (LB) medium until the optical density reached a value of 0.5. Protein expression was induced with 0.4 mM IPTG and bacteria were cultivated for 12 h at 25°C. Bacterial pellets were lysed with BugBuster Reagent (Merck 71456-3, Merck, Darmstadt, Germany). MBP-CRL1 and MBP-4ZF were affinity-purified on amylose resin from the soluble protein fraction according to the manufacturer's instructions (NEB, Ipswich, MA, USA). The protein concentration was determined by Bradford's method (Bradford, 1976). CRL1ΔN108 was similarly expressed in *E. coli*, purified by affinity chromatography on protein A, and used to generate rabbit antiserum. SDS-PAGE and Western blot were performed according to Breitler et al. (2001).

EMSA and SELEX experiments

For EMSA, probes were PCR-amplified from the forward single-stranded DNA as matrix (5′-CGTGGACTCACTACTGATCAG TXXXXXXTACATATCCTAAGTTGTCACCTAC-3′, where XXXXXX is the 6-bp specific motif) with the EMSA-forward (5′-CGTGGAC TCACTACTG-3′) and EMSA-reverse (5′-GTAGGTGACAACTTAGGA-3′) primers. The PCR program was as follows: 3 min at 94°C, followed by 20 cycles at 94°C for 30 sec, 40°C for 30 sec, and 72°C for 30 sec and a final extension step at 72°C for 1 min. The PCR product was analyzed on a 3% (w/v) agarose gel, purified with a Zymoclean DNA Gel Recovery kit (Zymo research D4001T, Zymo research, Irvine, CA, USA), and quantified. Next, 50 ng of DNA was 3′-end labeled with T4 DNA-polynucleotide kinase (NEB M0201S, Ipswich, MA, USA) and γ-$_{32}$P-ATP and purified on a Sephadex G-50 column (Amersham, GE Healthcare, Buckinghamshire, UK). Next, 8 ng of labeled DNA was mixed with 1 or 2 μg protein and 1 μg polydAdT in 20 μl binding buffer (5% [v/v] glycerol, 0.01 μg bovine serum albumin, 40 μM $ZnCl_2$, 150 mM NaCl, 20 mM Tris-HCl, pH 8). After 30 min of incubation at 20°C, mixtures were resolved on a 5% (w/v) acrylamide (3.3% [w/w] acrylamide/bis-acrylamide) gel. Gel was dried and exposed overnight on Hyperfilm MP (Amersham, GE Healthcare, Buckinghamshire, UK).

For SELEX the N18 degenerated DNA sequence (5′-CG TGGACTCACTACTGNNNNNNNNNNNNNNNNNNTCCTAATTGTC ACCTAC-3′) was synthetized and used for EMSA as described previously. The shifted protein/DNA complex was excised from the gel in reference to the corresponding autoradiography and incubated under agitation at 35°C overnight in 500 μl of elution buffer (0.5 M NH_4 acetate pH 8, 1 mM EDTA, 0.1% [w/v] SDS). The solution was treated with chloroform and DNA was precipitated with 3 M sodium acetate, pH 5 (0.2 v/v) and isopropanol (v/v). After centrifugation at 10 000 ***g*** during 30 min, the pellet was air-dried and dissolved in 20 mM Tris-HCl, pH 8. DNA was PCR-amplified with the EMSA-forward and -reverse primers and used to prepare the

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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 *CRL1* cDNA sequence without stop codon from a plasmid (Coudert et al., 2015). Primer sequences and PCR conditions are given in Table S3. The PCR amplification product was cloned into pENTR-D TOPO (Invitrogen K240020, Invitrogen, Carlsbad, CA, USA) and recombined into the Gateway binary vector pMDC83 using LR clonase (Invitrogen 11791100, Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. This yielded a CRL1-GFP fusion under the control of the CaMV 35S promoter. The corresponding plasmid was multiplied in *E. coli* strain TOP10 (Invitrogen C404010, Invitrogen, Carlsbad, CA, USA).

A cassette comprising *CRL1* (*LOC_Os03g05510*) and *AS2* (*At1g65620*) cDNA between the CaMV 35S promoter and the T-NOS terminator was excised by *Eco*RI and *Hin*dIII double enzymatic digestion from plasmid containing *CRL1* (Coudert et al., 2015) and pMDC32-AS2 and subcloned into the pB2KSP vector (Addgene, Cambridge, MA, USA) using the same restriction sites. Gel purification was done for both enzymatic digestion products using the Zymoclean DNA Gel Recovery kit (Zymo research D4001T, Zymo research, Irvine, CA, USA) according to the manufacturer's instructions. An open pB2KSP vector was dephosphorylated using calf intestinal alkaline phosphatase (CIAP) (Promega M1821, Promega, Madison, WI, USA). The *pro35S-CRL1-Tnos* and the *pro35S-AS2-Tnos* cassettes were ligated with the open pB2KSP using T4 DNA ligase (Promega M1801, Promega, Madison, WI, USA) and transferred for multiplication into *E. coli* strain TOP10 (Invitrogen C404010, Invitrogen, Carlsbad, CA, USA).

Two cDNA sequences corresponding to putative targets of CRL1 were obtained from the cDNA-KOME database (Kikuchi et al., 2003): *OsROP* (interPro: AK107862; cDNA-KOME: 002-134-B10) and *OsbHLH044* (AK107555; 002-130-B03). They were amplified using a specific couple of primers associated with BP flanking sequences for further cloning (Table S3). All PCR products were first cloned into the pGEM-T Easy vector (Promega A1360, Promega, Madison, WI, USA). Then, *OsROP* and *OsbHLH044* were recombined using BP clonase (Invitrogen 11789020, Invitrogen, Carlsbad, CA, USA) into the Gateway binary vector pC5300.OE (Khong et al., 2015). For all constructs, successful cloning was confirmed by sequencing.

Promoter cloning for transient transformation

Reverse and forward DNA fragments containing the *LBD-box* and *CRL1-box* tetramers or their corresponding mutants were synthetized by GenScript (Piscataway, NJ, USA) and annealed to obtain double-stranded DNA with appropriate restriction enzyme cohesive ends for further cloning (Table S4). The corresponding DNA molecules were directionally integrated using *Xba*I and *Sal*I restriction sites in the pGusSH-47 plasmid (Pasquali et al., 1994). Before ligation, the tetramers were phosphorylated using T4 polynucleotide kinase (Promega M1821, Promega, Madison, WI, USA) and the cleaved pGusSH-47 was dephosphorylated using CIAP (Promega M1821, Promega, Madison, WI, USA) according to the manufacturer's instructions.

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*) (*pOsROP*, *pOsHOX17*, *pROC4*, and *pOsbHLH044*, respectively) or the 1500-bp promoter fragment of *QHB* (*LOC_Os01g63510*; *pQHB*) using specific primers with enzymatic restriction sites (Table S3). The 1000-bp *OsHOX14* promoter fragment (*pOsHOX14*) was synthetized by GenScript (GenScript, Piscataway, NJ, USA) using DNA sequences retrieved from RAP-DB (Os07t0581700) (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 & MacHanick, 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 disinfection, hulled rice cv. Kitaake seeds were incubated under dark sterile conditions in pots containing half-strength Murashige and Skoog (MS/2) 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] macerozyme R10 [Duchefa M8002, Duchefa Biochemie B.V., Haarlem, the Netherlands]), followed by incubation in dark conditions for 4 h at 28°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 *LBD-box* and *CRL1-box* tetramers or (2) *pOsROP*, *pOsHOX17*, *pROC4*, *pOsHOX14*, *pQHB*, or *pOsbHLH044* in the pCAMBIA5300 vector, (ii) the empty *pRT101* effector plasmid or the *pRT101* plasmid carrying *CRL1* or *AS2* cDNA under the control of a CaMV 35S promoter, and (iii) the p2rL7 normalization plasmid (De Sutter et al., 2005) carrying the *LUC* gene under the control of the CaMV 35S 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.

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CRL1-GFP fluorescence localization was observed with an Axio Imager Z2 microscope (Zeiss, Marly-le-Roi, France) coupled to an X-cite 120 LEBBOOST LED illumination system (EXCELITAS Technologies, Waltham, MA, USA) and an Axiocam 506 color camera (Zeiss, Marly-le-Roi, France). GFP was excited at 457–487 nm and emission was detected at 502–538 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 Gateway binary vector pOs_Cas9_hpt (Miao et al., 2013). We used two sgRNAs framing the target gene to favor large mutation events in order to knock out the protein. For stable gene overexpression, plants were transformed using the binary vector pC5300.OE-*OsROP* or -*OsbHLH044* previously transferred into the *Agrobacterium tumefaciens* strain EHA105 (Khong et al., 2015). Taichung 65 and the *crl1* mutant in the Taichung 65 background were used for gene overexpression and Kitaake was used for CRISPR/Cas9-mediated mutagenesis; both cultivars were genetically transformed as previously described (Sallaud 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 *crl1* mutant background, a non-transgenic T2 line (without T-DNA) was kept as control (null sister *N_Os_crl1OE* lines). For *osrop cas* and *osbhlh044 cas* plants transformed by CRISPR/Cas9 mutagenesis, T0 were genotyped by PCR using primers flanking the two sgRNA positions (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 *crl1* 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 *Spe*I/*Xho*I 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*, *osbhlh044 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 (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%. 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) with 3.5 g per liter of plant agar (Duchefa P1001, Duchefa Biochemie B.V., Haarlem, the Netherlands). Embryos were oriented to allow growth of straight plantlets. For gain-of-function experiments, we selected for each gene three *Os_crl1OE* transgenic lines and their null sister *N_Os_crl1OE* line. More than 900 plantlets divided in subsets of at least fifty 22-day-old plantlets grown *in vitro* in glass tubes (ref. 26.131.36.09, Schott Duran, Wertheim, Germany) for each line were phenotyped to determine the number of crown roots. For loss-of-function experiments, we selected three *osrop cas* and *osbhlh044 cas* homozygous knockout mutant lines without the T-DNA carrying the *Cas9* coding sequence and presenting different mutations. More than 200 plantlets divided in subsets of at least thirty 13-day-old plantlets grown *in vitro* in glass tubes (ref. 26.131.36.09, Schott Duran, Wertheim, Germany) for each line were phenotyped to determine the number of crown roots. The root system of each phenotyped plantlet was removed from the tube and washed, and crown roots were counted.

For late vegetative stage phenotyping experiments, *Os*-TC65OE lines were grown in net houses of the Agricultural Genetics Institute of Hanoi in summer 2020. Plants were grown using sand columns (15 cm diameter × 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 pC5300.OE-*OsROP* or -*OsbHLH044* overexpression (*OsROP_crl1OE* or *OsbHLH044_crl1OE*, respectively) lines using their null sister lines (*N_OsROP_crl1OE* or *N_OsbHLH044_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 RNeasy Kit (Qiagen 74904, Qiagen, Venlo, the Netherlands) in the presence of DNase 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

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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 (N0) was taken as measured value. The expression level of the *OsEXP* (LOC_*Os06g0214100*) housekeeping gene was used to normalize gene expression between the different samples (Caldana et al., 2007; Coudert et al., 2015).

Array hybridization and analysis

All array hybridization steps were performed at the Transcriptomics platform of Montpellier University Hospital (CHU Montpellier, France). Total RNA quality control was performed on a Bioanalyzer 2100 (Agilent, Santa Clara, CA, USA). Sample preparation, one-round cDNA synthesis, and array hybridization on Gene Rice (JP) 1.1 ST chips were performed on the GeneAtlas system according to the manufacturer's instructions (Affymetrix, Santa Clara, CA, USA). Probe intensities were normalized using Robust Multi-array Average (Irizarry et al., 2003) as implemented in Expression Console software version 1.4.1.46 (Affymetrix, Santa Clara, CA, USA). Base 2 antilogarithm transformation was applied to probe set-level values before use.

DEGs between the *OsbHLH044_crl1OE* and *N_OsbHLH044_crl1OE* lines were detected using Transcriptome Analysis Console version 4.0 (Affymetrix) using a fold change cutoff of 1.5 and a *P*-value cutoff of 0.01.

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. Base 2 antilogarithm transformation was applied to probe set-level values before use.

ChIP-qPCR assay

Disinfected hulled DXCH seeds were sown in Petri dishes 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 incubated for 2 days in dark conditions at 28°C (ref. 82.1473.001, Starstedt, Nümbrecht, Germany) and for 3 days under a 14/10 h light/dark photoregime at 27°C. Obtained 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) (80–90 plantlets per Erlenmeyer) and grown for 24 h in the same conditions. Plantlets were then treated with DEX (ref. D4902, Sigma-Aldrich, MO, USA, 10 mM in ethanol) at a final concentration of 5 μM to induce the expression of CRL1-HA. Three independent biological replicates before (T0) and at 3 h (T3) or 9 h (T9) after DEX treatment were included. After 3 and 9 h of DEX treatment, stem bases were collected at appropriate time points and immediately frozen in liquid nitrogen for further chromatin extraction.

ChIP was performed with the EpiQuick Plant ChIP kit (Epigentek, NY, USA) following the manufacturer's instructions. Stem base chromatin components were cross-linked with 1% (v/v) formaldehyde under vacuum for 10 min until the stem bases were completely water-soaked. By adding glycine at a final concentration of 0.125 M, cross-linking was quenched. Cross-linked samples were then immediately ground to a powder in liquid nitrogen. The nuclei were isolated and lysed. Subsequently, the chromatin solutions were sheared by sonication with a Bioruptor® Pico sonication system (Diagenode, NJ, USA). The sheared chromatin solutions were centrifuged at 30 600 *g* for 10 min at 4°C. The clear supernatant lysates were then immunoprecipitated using an anti-HA-tag monoclonal antibody (ThermoFisher Scientific, MA, USA) to isolate the CRL1-HA–DNA complexes. The isolated DNA fragments were reverse cross-linked and purified using the EpiQuick Plant ChIP kit following the manufacturer's instructions. The purified input DNA ($_{IP}$DNA) which was not immunoprecipitated and ChIPed DNA ($_{ChIP}$DNA) which was immunoprecipitated were subsequently used for qPCR.

To determine the enrichment of regions bounded by CRL1-HA in the promoters of *OsROP* and *OsbHLH044*, ChIP-qPCR was performed using $_{IP}$DNA and $_{ChIP}$DNA from the ChIP preparation. For ChIP-qPCR data generation, Brilliant III Ultra-Fast qPCR Master Mix was used (Agilent, Santa Clara, CA, USA) with ROX normalization dye. Three technical replicates were conducted for each $_{IP}$DNA and $_{ChIP}$DNA from three independent biological replicates. To amplify the *LBD-box*- and/or *CRL1-box*-containing regions in the promoters of *OsROP* and *OsbHLH044*, specific primer pairs were designed (Table S3). The ChIP-qPCR data were normalized and relative RNA expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method. The ΔCt, ΔΔCt, and fold change values were calculated as follows:

$$\Delta Ct = {}_{ChIP}\text{DNA Ct} - {}_{IP}\text{DNA Ct},$$

$$\Delta\Delta Ct = \Delta Ct\ T3 \text{ or } T9 - \Delta Ct\ T0,$$

$$\text{fold change} = 2^{-\Delta\Delta Ct},$$

where ΔCt was calculated by normalizing the $_{ChIP}$DNA Ct values to $_{IP}$DNA values for each ChIP sample. The value of ΔΔCt 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 Dounce tissue grinder set (D9938, Sigma-Aldrich, MO, USA). The cell solutions in the CP3C buffer were filtered using two layers of Miracloth (Merck Millipore, MA, USA) and centrifuged at 2500 *g* for 20 min to harvest the nuclei. The nuclei were washed in EpiQuick Plant ChIP kit (Epigentek, NY, USA) CP3D buffer and pelleted by centrifugation at 2500 *g* for 1 min. The pellet containing nuclei was suspended in 80 μl of protein extraction buffer (20 mM Tris-HCl, pH 7.4, 100 mM NaCl, 10 mM $Na_2EDTA{\cdot}2H_2O$, 25 mM D-glucose, 5 mM EGTA, 5% [v/v] glycerol, 5 mM DTT). Subsequently, 10 μL of 5× Laemmli sample buffer (60 mM Tris-HCl, pH 6.8, 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

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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 *crl1* 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 *crl1* 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 *crl1* 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 *crl1* mutant background.

Table S3. List of primers used.

Table S4. Sequence of the *cis*-regulatory tetramers.

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