The URL1–ROC5–TPL2 transcriptional repressor complex represses the ACL1 gene to modulate leaf rolling in rice

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Abstract

Moderate leaf rolling is beneficial for leaf erectness and compact plant architecture. However, our understanding regarding the molecular mechanisms of leaf rolling is still limited. Here, we characterized a semi-dominant rice (Oryza sativa L.) mutant upward rolled leaf 1 (Url1) showing adaxially rolled leaves due to a decrease in the number and size of bulliform cells. Map-based cloning revealed that URL1 encodes the homeodomain-leucine zipper (HD-Zip) IV family member RICE OUTERMOST CELL-SPECIFIC 8 (ROC8). A single-base substitution in one of the two conserved complementary motifs unique to the 3'-untranslated region of this family enhanced URL1 mRNA stability and abundance in the Url1 mutant. URL1 (UPWARD ROLLED LEAF1) contains an ethylene-responsive element binding factor-associated amphiphilic repression motif and functions as a transcriptional repressor via interaction with the TOPLESS co-repressor OsTPL2. Rather than homodimerizing, URL1 heterodimerizes with another HD-ZIP IV member ROC5. URL1 could bind directly to the promoter and suppress the expression of abaxially curled leaf 1 (ACL1), a positive regulator of bulliform cell development. Knockout of OsTPL2 or ROC5 or overexpression of ACL1 in the Url1 mutant partially suppressed the leaf-rolling phenotype. Our results reveal a regulatory network whereby a transcriptional repression complex composed of URL1, ROC5, and the transcriptional corepressor TPL2 suppresses the expression of the ACL1 gene, thus modulating bulliform cell development and leaf rolling in rice.
Introduction

Leaf size and shape are important components of plant architecture (Li et al., 2010). Appropriate leaf rolling maximizes light capture and reduces transpiration under dry conditions (Lang et al., 2004; Moon and Hake, 2011). Bulliform cells are a group of specialized epidermal cells on the adaxial leaf blade surface in monocots, like rice, that control leaf rolling (Itoh et al., 2005; Xiang et al., 2012). To date, several rice (Oryza sativa L.) genes have been identified to regulate abaxial or adaxial leaf rolling by altering the number or size of bulliform cells. Overexpression of abaxially curled leaf 1 (ACL1) increases the number and exaggerates the size of bulliform cells, resulting in abaxially rolled leaves (Li et al., 2010). Similarly, overexpression of the zinc finger homeodomain (HD) class transcription factors OsZHD1 and OsZHD2 also induced the abaxial leaf rolling due to the increased number of bulliform cells (Xu et al., 2014). The rice adaxialized leaf1 (adj1) mutant shows abaxially rolled leaves in which large bulliform-like cells are present on either side of leaf (Hibara et al., 2009). In the loss-of-function mutant of RL14 which encodes a 2OG-Fe oxygenase, altered composition of the secondary cell wall results in water deficiency and shrinkage of bulliform cells leading to incurved leaves (Fang et al., 2012).

The plant-specific HD-leucine zipper (HD-Zip) proteins can be subdivided into four subfamilies: HD-Zip I–IV based on distinct protein sequence features and functions (Elhiti and Stasolla, 2009). There are 16 HD-Zip IV genes in the Arabidopsis genome, of which at least three, namely, ARABIDOPSIS THALIANA MERISTEM LAYER1 (ATML1), PROTODERMAL FACTOR2 (PDF2), and HOMEODOMAIN GLABROUS11 (HDG11), play pivotal roles in differentiation of the epidermis during both embryonic and postembryonic development (Nakamura et al., 2006). Similarly, the maize (Zea mays) HD-Zip IV gene Outer Cell Layer1 (OCL1), is critical to specify embryo protoderm identity and maintain the L1 layer of cells in the shoot apical meristem (SAM; Depege-Fargeix et al., 2011). In rice, there are at least nine HD-Zip IV family genes, named Rice Outermost Cell-specific 1–9 (ROC1–9), which exhibit an epidermis-specific expression pattern (Ito et al., 2003). ROC5 has been further demonstrated to negatively regulate bulliform cell development and modulate leaf rolling (Zou et al., 2011).

The expression of many genes is regulated at the post-transcriptional level through mRNA degradation or stabilization, and the 3′-untranslated region (3′-UTR) of a gene plays an important role in mediating this process (Ross, 1995). Some inherently unstable mRNAs contain a specific motif, that is, the AU-rich elements (ARE; Tavares et al., 2000) in the 3′-UTR. Binding of the ARE-binding proteins elicits rapid degradation of mRNA (Vlasova and Bohjanen, 2008; Simone and Keene, 2013). In addition, the stem-loop domain within a 3′-UTR also modulates gene expression (Holden and Harris, 2004). Potential stem-loop structures are predicted within the 3′-UTR of numerous HD-ZIP IV family members in Arabidopsis, rice, and maize (Javelle et al., 2011; Zalewski et al., 2013; Burgess and Freeling, 2014). There are two evolutionarily conserved motifs of 19 and 21 nucleotides (nt) within the 3′-UTR of HD-ZIP IV genes. Importantly, these two motifs are partially complementary, facilitating the formation of a stem-loop structure via base pairing between the two motifs. However, involvement of these two motifs in maintaining proper expression levels of HD-ZIP IV family genes has not been verified experimentally.

In this study, we report the URL1 gene encoding the HD-Zip IV family member ROC8, which regulates leaf rolling in rice. A single nucleotide substitution in one of the two conserved motifs in the 3′-UTR enhances URL1 mRNA stability in the Url1 mutant. URL1 interacts physically with ROC5, another HD-Zip IV family member, and TOPLESS (TPL) corepressor OsTPL2 through its ethylene-responsive element binding factor-associated amphiphilic repression (EAR) motif, which leads to suppressed expression of the downstream gene ACL1 and inhibited bulliform cell development. These data reveal the important role of URL1 in leaf shape configuration and the potential role of the two conserved motifs in 3′-UTR in fine-tuning HD-ZIP IV mRNA stability.

Results

The rice semi-dominant mutant Url1 shows adaxially rolled leaves due to reduced number and size of bulliform cells

To study the underlying mechanisms of rice leaf rolling, we screened the ethyl methanesulphonate-induced mutant population of a japonica variety Nipponbare and identified the Url1 mutant with visibly incurved leaves. The Url1 mutant had adaxially rolled leaves from the seedling stage, which became more obvious as the rice plants developed (Figure 1). The F1 plants derived from a cross between Url1 and wild type (WT) had semi-rolled leaves, an intermediate between the flat and fully rolled leaves of the homozygous parental plants (Figure 1, A and C). The leaf-rolling index (LRI) was used to quantify the extent of leaf rolling. While the LRI of WT leaves was close to 0, the LRI values were 63%±2.72% in the Url1 mutant and 39%±5.23% in the F1 heterozygote (Figure 1N). In the F2 population, plants with flat, semi-rolled, and rolled leaves had a segregation ratio of 1:2:1 (32:50:25, χ² = 1.37, P > 0.05), indicating that the rolled leaf character of Url1 is caused by a semi-dominant mutation.

Further, bulliform cells of the leaf from 50-d-old plants were stained using toluidine blue O (TBO; Dudley and Poethig, 1993; Hernandez et al., 1999; Li et al., 2010). Bulliform cells stained purple were arranged in three columns along the large vein and small vein in WT. In contrast, only two columns were observed in the Url1 mutant, although the linear patterning of bulliform cells on the leaf blade did not alter (Figure 1, D–G). To observe bulliform cells and other cell types more clearly, paraffin cross-sectioning of mature leaves was undertaken. Typically, bulliform cells were arranged in groups of 6.67±1.18 adjacent cells near the large veins and 6.88±0.72 near the small veins.
Figure 1 The phenotype of *Url1* mutant. A–C, In mature plants, the leaves rolled adaxially like a cylinder in the *Url1* mutant, semi-rolled in *F*<sub>1</sub>, compared with the flat leaves in WT. Bars = 10 cm in (A), 5 cm in (B), and 200 μm in (C). D–G, Toluidine blue O staining of bulliform cells which were stained in purple. Bars = 50 μm. H–M, Cross sections of WT and *Url1* mature leaves. Bars = 100 μm in (H) and (K), 50 μm in (I), (J), (L), and (M). Ad, adaxial; Ab, abaxial; Lv, large vein; Sv, small vein; Bc, bulliform cell; N, LRI of WT and *Url1* are shown. Data are presented as mean ± se (*n* = 15). Significance of data is tested by Student’s *t* test (**P* < 0.01). O and P, Bulliform cells number (O) and area (P) of WT and *Url1* are shown. Data are presented as mean ± se (*n* = 10). Significance of data is tested by Student’s *t* test (**P* < 0.01).
in WT, whereas the number was significantly reduced to 5.19 ± 0.75 and 4.77 ± 0.83, respectively, in the Ur1 mutant (Figure 1O). Furthermore, the size of bulliform cells was also reduced. The total area of bulliform cells adjoining large veins was reduced by 42.63% in Ur1 (1,768.44 ± 611.25 μm²) compared with that in WT (3,082.83 ± 588.17 μm²), and reduced by 57.66% for those neighboring small veins (966.66 ± 386.76 μm² in Ur1 versus 2283 ± 377.43 μm² in WT; Figure 1P). Other cell types, such as sclerenchymatous cells at both sides of the large and small veins remained normal in Ur1 (Supplemental Figure S1). Thus, both the number and size of bulliform cells were reduced in the Ur1 mutant, which is responsible for the adaxial leaf rolling.

**URL1 encodes the HD-ZIP class IV transcription factor ROC8**

The **URL1** locus was primarily mapped between two InDel markers M1 and M6 on the short arm of chromosome 6 and further narrowed to a 53-kb region delimited by two markers M3 and M4 (Figure 2A). Three genes were annotated within this region by the Rice Genome Annotation Project (http://rice.plantbiology.msu.edu/). Sequence comparison revealed a C-to-T substitution at the 679th nt after the stop codon of LOC_Os06g10600 (Figure 2A). Using the 3′- and 5′-rapid amplification of cDNA ends (RACE) technique, full-length cDNA sequence of LOC_Os06g10600 was determined, which has a long 3′-UTR of 732 nt. Therefore, the mutation site is located within the 3′-UTR of LOC_Os06g10600. As the 3′-UTR plays an important role in the post-transcriptional regulation of gene expression (Ross, 1995), we examined the expression level of LOC_Os06g10600. The transcript level of LOC_Os06g10600 increased significantly in Ur1 leaves and also moderately in the F₁ heterozygote (Figure 2B), consistent with the semidominant inheritance pattern. Therefore, LOC_Os06g10600 is designated as **URL1** hereafter.

The **URL1** gene consists of 10 exons and 9 introns (Figure 2A), encoding the 710-residue HD-ZIP IV family transcription factor ROC8 (Supplemental Figure S2). **URL1** contains four typical domains of HD-ZIP IV proteins: an N-terminal HD, followed by a leucine-zipper motif (ZIP), a steroidogenic acute regulatory protein-related lipid transfer (START) domain, and a START-adjacent domain at the C-terminus (Figure 2C). Phylogenetic analysis showed that **URL1** is the closest to Arabidopsis HOMEODOMAIN GLABROUS11 (HDG11) and HDG12, maize Outer Cell Layer15 (OCL15) and OCL17, and rice RICE OUTERMOST CELL-SPECIFIC6 (ROC6; Supplemental Figure S3).

**Increased expression of URL1 is responsible for the rolled leaves phenotype in Ur1**

To verify whether the point mutation in the 3′-UTR of **URL1** is responsible for the **Ur1** phenotype, a 6,070-bp genomic DNA fragment of the **URL1** gene from the **Ur1** mutant was introduced into WT. The transgenic lines obtained displayed the adaxially rolled leaf phenotype with increased **URL1** transcript level, mimicking the leaf-rolling phenotype of the **Ur1** mutant (Figure 2, D, F, and G). Additionally, the RNAi construct targeting the 3′-UTR of **URL1** was introduced into the **Ur1** mutant. As expected, reducing **URL1** expression in the **Ur1** mutant markedly suppressed the adaxial rolling of leaf in the transgenic lines (Figure 2, E, H, and I). Furthermore, we generated **URL1** knockout mutants through the CRISPR-Cas9 approach in the **Ur1** mutant background (Ma et al., 2015). These **URL1** loss-of-function mutants exhibited abaxially rolled leaves with increased number and size of bulliform cells (Supplemental Figure S4). Therefore, the phenotypes of both gain- and loss-of-function mutants collectively indicated that **URL1** is a key regulator of leaf rolling via bulliform cell development control.

**Mutation in the conserved motif in 3′-UTR enhances the stability of **URL1** mRNA**

Next, we aimed to explain why the point mutation in the 3′-UTR of **URL1** results in increased transcript level. Like most members of the HD-ZIP IV family, the 3′-UTR of **URL1** harbors two evolutionarily conserved motifs, i.e., CNS1 (conserved noncoding sequence 1) and CNS2 (Javelle et al., 2011; Burgess and Freeing, 2014). The C-to-T substitution at the 679th nucleotide after the stop codon resides within the CNS2 motif (Figure 3, A and C). CNS1 and CNS2 are partially complementary to each other and the base pairing between them mediates the formation of a stem-loop structure as predicted by the software RNAfold (Gruber et al., 2008). The C679T substitution changed the G–C base pairing to G–U mismatch, which may reduce the stability of stem-loop structure (Supplemental Figure S5).

As the stem-loop structure in the 3′-UTR has been characterized as an mRNA destabilizing element (Paschoud et al., 2006; Mullen and Marzluff, 2008; Leppke et al., 2013; Chang et al., 2016), we examined potential cleavage sites in the 3′-UTR of **URL1**. Using the RNA ligase-mediated 5′-rapid amplification of cDNA ends (RLM-5′RACE) technique, two fragments of about 300 and 100 bp were obtained (Figure 3B). Amplification of the cleaved fragment of OsSPL14 by mRNA156 was used as a positive control (Jiao et al., 2010). Cloning and sequence analysis of the 300-bp fragment revealed six cleavage sites within or surrounding the CNS1 motif. Analysis of the 100-bp fragment revealed one cleavage site at the 5′-boundary of CNS2 (Figure 3C). To detect the effects of cleavage sites on the **URL1** mRNA stability, we designed three primer sets located within the CDS region (**URL1**-I), 3′-UTR region upstream of CNS1 (**URL1**-II), and 3′-UTR region encompassing both CNS1 and CNS2 (**URL1**-III) to perform reverse transcription quantitative PCR (RT-qPCR) analysis (Figure 3A). All three primer sets detected a significant increase in the **URL1** expression in the **Ur1** mutant, wherein **URL1**-III flanking the cleavage sites detected the most pronounced increase (Figure 3D). To explore the possible mechanisms of **URL1** mRNA...
accumulation in the Url1 mutant, we first performed a nuclear run-on RT-qPCR (Folta and Kaufman, 2006; Patrone et al., 2000; Roberts et al., 2015) to quantify the nascent transcripts of URL1 in the Url1 mutant and WT using the three primer sets P1, P2, and P3 spanning the first, fourth, and fifth intron–exon boundary, respectively (Figure 3A). The results showed that there was no significant difference in nascent transcription between WT and the Url1 mutant (Figure 3E), suggesting that the C679T mutation did not affect nascent transcription and that URL1 mRNA levels may
be modulated post-transcriptionally. We then compared the decay rate of the WT and mutant URL1 mRNA using RT-qPCR. Upon treatment with cordycepin, which inhibits transcription (Hori and Watanabe, 2008), the URL1 transcript level was detected at 1-h intervals. The mutated URL1 (C679T) degraded much slower than the WT URL1 (Figure 3F), suggesting that the mutant URL1 mRNA has increased stability. These results confirmed that the stem-loop structure in the 3'-UTR triggers degradation of URL1 mRNA.

To corroborate that the 3'-UTR of URL1 was involved in mRNA destabilization, we used a firefly luciferase-based reporter system (Figure 3G; Ruberti et al., 1991). The 3'-UTR

Figure 3 A single-base substitution in the conserved complementary motifs unique to 3’-UTR of this family enhanced the URL1 mRNA stability. A, Position of the two conserved motifs CNS1 (19 nt) and CNS2 (21 nt) in the 3’-UTR of URL1. URL1-I, URL1-II, and URL1-III indicate the locations of primer sets used in (D). P1, P2, and P3 indicate the locations of primer sets used in (E). B, 5’-RLM-RACE showed that two putative cleavage products of about 300 and 100 bp (red triangle and pentagram) were present in both WT and Url1. Amplification of miRNA156 cleavage product of OsSPL14 was used as a positive control. C, The cleavage sites detected within 3’-UTR of URL1. Sixteen and 18 independent clones were sequenced for the large and small cleavage product, respectively. Arrows indicate cleavage sites relative to the CNS1 or CNS2 motif. D, RT-qPCR analysis of the URL1 mRNA transcript level in the Url1 mutant and WT using primer sets URL1-I, URL1-II, and URL1-III located in different regions of URL1, respectively. The rice ubiquitin gene was used as an internal control and the URL1 expression level in the Url1 mutant was normalized to the WT. Data are presented as mean ± se (n = 4). Significance of data is tested by Student’s t test (**P < 0.01). E, Quantification of nascent URL1 transcripts by biotin-16-UTP immunocapture nuclear run-on RT-qPCR in WT and Url1. RT-qPCR analysis was done with three primer sets P1, P2, and P3 which span the first, fourth, and fifth intron–exon boundary, respectively. F, The URL1 mRNA decay assay in the Url1 mutant and WT. URL1 mRNA level was determined by RT-qPCR analysis after treatment with cordycepin for 0, 1, 2, 3, 4, and 5 h. URL1 expression levels before cordycepin treatment (0 h) in WT and the Url1 mutant are set as 1.0, respectively. Data are presented as means ± se (n = 5). G, Schematic representation of 3'-UTR constructs used in the luciferase assay. H and I, Relative luciferase activities (H) and mRNA level (I) were measured when 3'-UTR, m3'-UTR, Δ3'-UTR of URL1 were inserted into the P35S::LUC reporter vector. Data are presented as mean ± se (n = 4), and the same letters above each bar indicate that means did not differ significantly at the 0.05 level in Tukey’s multiple comparison test.
of URL1 from both WT and the Url1 mutant was transcriptionally fused to the 3’-end of the LUCIFERASE (LUC) ORF. A fourth reporter construct was also created in which the CNS2 motif was deleted. The four LUC reporter constructs along with the internal control (Renilla luciferase, RLUC) were co-transformed into rice leaf protoplasts. Luciferase activity in the cells transformed with LUC-3’-UTR was significantly lower than that in those transformed with the control LUC construct. However, cells transformed with two mutated versions of 3’-UTR (LUC-3’UTR∆CNS2 and LUC-3’UTR∆∆CNS2) showed significantly increased luciferase activity compared to those transformed with the intact 3’-UTR (LUC-3’UTR; Figure 3H). RT-qPCR assay on the same samples revealed that LUC mRNA level correlated well with the luciferase activity. The presence of 3’-UTR significantly reduced the LUC mRNA level, whereas expression of LUC-3’UTR∆CNS2 and LUC-3’UTR∆∆CNS2 resulted in partial restoration of LUC mRNA level (Figure 3I). These results suggest that the 3’-UTR of URL1 is responsible for its mRNA stability.

Finally, we confirmed the effect of C679T substitution on URL1 mRNA stability in planta. We generated transgenic rice plants expressing URL1 cDNA with the WT or mutated 3’-UTR driven by the rice Actin1 promoter. All 14 transgenic lines of the WT URL1 cDNA (B197) showed flat-leaf phenotypes in the T0 generation. However, 10 out of 12 lines transformed with the mutant Url1 cDNA (B320) showed adaxial leaf rolling (Supplemental Figure S6A). RT-qPCR analysis of representative lines showed that the URL1 mRNA level was increased in the B320 lines but not in the B197 lines, consistent with their LRI (Supplemental Figure S6, B and C). These data indicate that the Url1 mutant mRNA was more readily accumulated than the WT URL1 mRNA, as a result of the enhanced mRNA stability.

Expression pattern and subcellular localization of URL1

RT-qPCR analysis revealed that URL1 was ubiquitously expressed in all tissues with a relatively higher expression in leaf, root, and stem. In particular, URL1 expression in younger leaves was significantly higher than that in the older leaves (Figure 4A). Histological staining of the URL1::GUS transgenic rice plants revealed strong GUS activity in the epidermal layer of leaf blade and sheath, although signal could also be detected in the inner tissues (Supplemental Figure S7, I and J). In the in situ hybridization analysis, URL1 mRNA was detected in the entire L1 layer of SAM, axillary meristem, leaf primordium, and young leaves (Figure 4, B–D and H; Supplemental Figure S7, L–N). Cross-sectional analysis of the young leaves revealed more intense signal in the epidermal cell layer and vasculature tissue (Figure 4, E–G and I–K). The URL1 expression pattern in the Url1 mutant was similar to that in WT. However, the signal intensity was greatly increased in the Url1 mutant (Figure 4, B–K), which is consistent with the enhanced stability of Url1 mutant mRNA. Further analysis showed that the URL1::GFP protein localized in the nucleus of rice protoplast (Figure 4, L and M), consistent with the function of HD-ZIP IV family proteins as transcriptional regulators.

URL1 recruits TPL2 with its EAR motif to function as a transcriptional repressor

The ethylene-responsive element binding factor-associated amphiphilic repression (EAR) motif is a transcriptional repression domain. Proteins containing this motif negatively regulate the expression of diverse genes (Kagale et al., 2010). A typical EAR motif (LDLIL) is located at the N-terminus of URL1 protein (Figures 2, C and 5, A), suggesting that URL1 may also function as a transcriptional repressor. Therefore, we measured its transcriptional activity using the luciferase transient expression assay in rice protoplasts. In the reporter construct, the LUC gene was driven by the CaMV35S promoter fused with five copies of GAL4-binding site (Aguilera et al., 2007). In the effector constructs, URL1 was fused in-frame with the GAL4 DNA-binding domain (GAL4DBD) or GAL4DBD-VP16 (Sadowski et al., 1988). To confirm the effect of the EAR motif on transcriptional activity, the last two conserved Leu residues were replaced by Ser residues (Figure 5B). Each of the six effector constructs with the reporter construct (Figure 5C) was co-transformed into rice protoplasts. As expected, LUC activity was strong in those protoplasts transformed with GAL4DBD or GAL4DBD-VP16. However, LUC activity was severely attenuated in protoplasts transformed with GAL4DBD-URL1 or GAL4DBD-URL1-VP16 (Figure 5D), indicating that URL1 functions as a transcriptional repressor. Mutation in the EAR motif (URL1mEAR) partially relieved this repression (Figure 5D).

The EAR motif can recruit the TPL family proteins, forming a transcriptional repression complex (Szemenyei et al., 2008; Pauwels et al., 2010). Thus, we examined potential interactions between URL1 and TPL2, the rice homolog of TPL, using yeast two-hybrid and firefly luciferase complementation imaging (LCI) assays. URL1 could interact with the N-terminal region of TPL2 in vitro and in vivo (Figure 5, E and F), which is known to be sufficient for mediating the interaction between TPL and EAR-containing proteins (Causier et al., 2012; Zhang et al., 2017). Mutation in the EAR motif (URL1mEAR) weakened the interaction between URL1 and TPL2 (Figure 5, E and F), suggesting that the interaction between URL1 and TPL2 depends on the EAR motif.

To ascertain the role of URL1 transcriptional repression activity in the regulation of leaf rolling, four tandem copies of the exogenous EAR motif were fused to the N-terminus of URL1 cDNA and expressed under the maize ubiquitin1 promoter (Figure 5G). The construct was transformed into the Url1 mutant. The transgenic lines displayed extremely...
Figure 4 Expression pattern and subcellular localization of the URL1 protein. A, RT-qPCR analysis of URL1 expression in various tissues including young leaf at the seedling stage, mature leaf at the heading stage, sheath, stem, root, panicle, glume, and seed. Rice ubiquitin was used as an internal control. Data are presented as mean ± se (n = 3). B–K, In situ hybridization to detect URL1 transcripts in WT and the Url1 mutant on the longitudinal section from the shoot apex region of 7-d-old rice seedling (B, C, D, H) and the cross section from young leaves of 7-d-old rice seedling (E, F, G, I, J, K). Black-dotted areas in (D) and (H) were magnified and inserted in the upper right corner, respectively. Black and red arrow heads indicate the in situ hybridization signals in the epidermal cells in young leaves and mature leaves, respectively, at both the abaxial and adaxial sides. Black-dotted areas in (F) and (J) were magnified and shown in (G) and (K), respectively. The sense probe was hybridized and used as negative controls in (B, C, E, I). Bars = 200 μm. L and M, Subcellular localization of the URL1 protein. Transient expression of URL1–GFP fusion protein in rice protoplasts revealed that URL1 is mainly located in the nucleus. p35S:GFP was used as the control. Bars = 20 μm.
adaxially rolled leaves (Figure 5, H and I). Observation of leaf cross-sections showed severe leaf rolling with one edge of leaf blade completely enwrapped by the other (Figure 5J). Besides, the EAR-URL1 transgenic plants were dwarf and sterile (Figure 5H). In contrast, fusion of the VP16 activation domain to URL1 had no effect on leaf rolling and plant height.

To clarify the relationship between URL1 and TPL2 in regulating leaf rolling, we created the TPL2 knockout mutants in the Url1 mutant background using CRISPR/Cas9 technology, and characterized two independent mutant alleles, tpl2-1/Url1 and tpl2-2/Url1 (Figure 6A). The adaxially rolled leaf phenotype of Url1 was partially alleviated in the tpl2-1/Url1 and tpl2-2/Url1 mutants (Figure 6B). The LRI values of tpl2-
1/Url1 and tpl2-2/Url1 were 32% and 36%, respectively, which were greatly reduced compared with that of the Url1 mutant (77%; Figure 6C). Further microscopic observations revealed that tpl2-1/Url1 and tpl2-2/Url1 had more and larger bulliform cells than Url1 (Figure 6, D–M). Therefore, the transcriptional corepressor activity of TPL2 is required for URL1-regulated adaxial leaf rolling in the gain-of-function Url1 mutant.
URL1 interacts directly with ROC5

ROC5, another member of the HD-ZIP IV family in rice, modulates leaf rolling via regulation of bulliform cell development (Zou et al., 2011). This prompted us to check whether URL1 and ROC5 interact with each other. Both yeast two-hybrid and co-immunoprecipitation (Co-IP) assays showed that URL1 was able to interact with ROC5 in vitro and in vivo, respectively (Figure 7A and C). It was also observed that URL1 and ROC5 themselves could form a homodimer in yeast (Figure 7A). The quantitation of β-galactosidase activity revealed that the strength of the URL1–ROC5 interaction was higher than the URL1–URL1 and ROC5–ROC5 interactions (Figure 7B). To further compare the interaction strength among the URL1–URL1, ROC5–ROC5, and URL1–ROC5 combinations in vivo, we performed LUC complementation imaging assays in Nicotiana benthamiana leaves (Figure 7D). Strong fluorescence was detected for the four coexpression combinations of NLUC-ROC5 + CLUC-ROC5, NLUC-URL1 + CLUC-URL1, NLUC-ROC5 + CLUC-URL1 and NLUC-URL1 + CLUC-ROC5 (Figure 7D). Quantitative analysis showed that the relative LUC activities were significantly higher in coexpression combinations of NLUC-ROC5 + CLUC-ROC5 and NLUC-URL1 + CLUC-ROC5 than those in coexpression combinations of NLUC-ROCS + CLUC-ROC5 and NLUC-URL1 + CLUC-URL1 (Figure 7E), suggesting that the URL1 and ROC5 heterodimer is more easily formed than the ROC5/ROC5 or URL1/URL1 homodimers in vivo.
To clarify their relationship at the genetic level, we crossed the \textit{Url1} mutant with \textit{oul1}, the T-DNA knockout mutant of \textit{ROCS} in Nipponbare background which shows abaxially rolled leaves (Zou et al., 2011). Knockout of \textit{ROCS} in the homozygous \textit{Url1} mutant (i.e. the \textit{Url1 oul1} double mutant) significantly reduced the degree of adaxial leaf rolling compared with the \textit{Url1} single mutant (Figure 8, A–C, the second and fifth sample). Interestingly, knockout of \textit{ROCS} in the heterozygous \textit{Url1} mutant (i.e. the \textit{Url1+/oul1} mutant) completely suppressed the adaxially semi-
rolled leaf phenotype and the leaf became as flat as WT (Figure 8, A–C, the third and sixth sample). Further microscopic observations revealed that both the number and size of bulliform cells increased modestly in the oul1 Url1 double mutants compared with that in the Url1 single mutant, and were restored to the WT level in the Url1+ oul1 mutant (Figure 8, D–Q). To further confirm their genetic interaction, we knocked out ROCS in the Url1 mutant using CRISPR/Cas9 technology and obtained two homozygous T2 mutants with a 1- and 2-bp deletion, respectively. The degree of adaxial leaf rolling was significantly reduced in both lines (Supplemental Figure S8). These results suggest that ROCS is required for the function of URL1 and that these two proteins probably function as a heterodimer that modulates leaf rolling. Meanwhile, in the absence of ROCS, URL1 may function as a homodimer that regulates leaf rolling.

**ACL1 is a putative target gene repressed by URL1**

Next, we aimed to identify the downstream target gene regulated by URL1. We analyzed the transcription levels of ZHD1, ADL1, ACL1, and RL14, which have been shown to regulate rice leaf rolling by modulating bulliform cell development (Hibara et al., 2009; Li et al., 2010; Fang et al., 2012; Xu et al., 2014). ACL1 was significantly downregulated, whereas ZHD1, ADL1, and RL14 were upregulated in the Url1 mutant (Figure 9A). Considering that URL1 functions as a transcriptional repressor and that Url1 is a gain-of-function mutant, ACL1 could be the potential target of URL1.

The L1 box (5′-TAAATGYA-3′) is a well-conserved cis-regulatory element in the promoter of target genes regulated by HD-ZIP IV transcription factors (Abe et al., 2003). Interestingly, two L1 boxes were identified at 1,846–1,853 and 2,600–2,607-bp upstream of the start codon of ACL1 (Figure 9B). Electrophoretic mobility shift assay (EMSA) revealed that glutathione-S-transferase (GST)-URL1 could remarkably reduce the electrophoretic mobility of the probes containing two ACL1 L1 boxes (Figure 9, C and D), but the GST protein alone could not cause mobility reduction. The binding specificity of URL1 to the L1 box was confirmed by effective competition using an excess amount of unlabeled probe and the absence of mobility shift of the mutated L1 box probes (Figure 9, C and D). Then chromatin immunoprecipitation (ChiP) was performed with the transgenic line B320-1 (Supplemental Figure S6A). DNA regions I and II of ACL1 (ACL1-I, ACL1-II), which contained the L1-box, were significantly enriched in the communoprecipitates from the transgenic line. In contrast, there was no enrichment for the negative control region of ACL1 (ACL1-III) or promoter region of the nontarget gene ubiquitin (Figure 9E). Therefore, URL1 could physically bind to the ACL1 promoter via the L1 box.

To examine whether ACL1 was transcriptionally repressed in response to URL1 expression in planta, we used a plINDEX two-component induction system (Figure 9F; Ouwerkerk et al., 2001). The URL1 transcript increased dramatically upon dexamethasone (DEX) treatment (Figure 9G). Accordingly, the expression level of ACL1 decreased distinctly upon the induction of URL1 expression (Figure 9H). In contrast, the noncandidate gene SLL1 (Zhang et al., 2009) was not evidently repressed by DEX treatment (Figure 9I). The expression levels of these genes were not obviously affected by the DEX treatment in WT rice plants (Supplemental Figure S9). These results indicate that URL1 can repress ACL1 gene expression.

To examine the functional relationship of ACL1 with URL1 in the modulation of leaf rolling, we overexpressed ACL1 under control of the rice Actin1 promoter in the Url1 mutant (B432). The adaxial rolling of the Url1 mutant leaf was evidently reduced by the overexpression of ACL1 (Figure 10A). The decreased LRI was correlated to the increased expression level of ACL1 in the transgenic lines (Figure 10, B and C). Since ACL1 regulates leaf rolling by controlling the number and size of bulliform cells (Li et al., 2010), we further made microscopic observation of the leaves in B432 transgenic plants. Both the number and size of bulliform cells was significantly increased in B432 when compared with those in the Url1 mutant, suggesting that overexpression of ACL1 could partially rescue the adaxially rolled leaves of Url1 via modulating bulliform cell development (Figure 10, D–M).

**Discussion**

**URL1 negatively regulates bulliform cell formation and development**

In this study, we identified URL1 as a transcriptional suppressor to modulate leaf rolling through mutant analysis. In the gain-of-function Url1 mutant, both the number and size of bulliform cells decreased and the leaf rolled adaxially (Figure 1). In contrast, in the CRISPR/Cas9-mediated Url1 knockout line, both the number and size of bulliform cells increased and the leaf rolled abaxially (Supplemental Figure S4). Therefore, our data strongly support that URL1 negatively regulates the formation and development of bulliform cells. However, localization of bulliform cells between two vascular bundles and polarized distribution of bulliform cells along the adaxial–abaxial axis did not change in Url1 (Figure 1). Consistent with an obvious phenotype at early growth stage, URL1 is predominantly expressed in young leaves rather than mature leaves (Figure 4; Supplemental Figure S7), indicating that URL1 is directly involved in the establishment rather than maintenance of bulliform cells in the adaxial epidermis.

Besides its role in bulliform cell development, URL1 may also regulate the development of other epidermal cells. First, URL1 expression is not specifically limited to the bulliform cells, but is also observed in the entire L1 layer of SAM, axillary meristem, leaf primordium, and young leaves (Figure 4, B–D and H; Supplemental Figure S7, L–N). Second, as evident in the semithin cross-sectional analysis of leaf blades, other epidermal cells...
besides bulliform cells also became smaller in the gain-of-function Url1 mutant (Supplemental Figure S1) and larger in the loss-of-function Url1 knockout line (Supplemental Figure S4, D–I and L), respectively. Therefore, URL1 possibly regulates the size of all cells in the L1 layer of leaves. However, these nonbulliform epidermal cells showed comparable changes in cell size at both the abaxial and adaxial sides of leaf blade (Supplemental Figure S1C), and thus had no effect on leaf rolling. In contrast, bulliform cells are present only

![Figure 9](image-url)
on the adaxial side of leaf blade and are highly linked to leaf rolling in rice and other grasses (O’Toole et al., 1979; Kadioglu and Terzi, 2007). Therefore, changes in the number and size of bulliform cells may largely account for the leaf-rolling phenotypes in Url1 gain-of-function mutant and URL1 knockout lines.

Potential roles of the two conserved motifs in 3′-UTR in fine-tuning HD-ZIP IV mRNA stability

Previous studies showed that most HD-ZIP IV genes in plants contain two evolutionarily conserved motifs in their 3′-UTR (Javelle et al., 2011; Zalewski et al., 2013; Burgess and Freeling, 2014). These two motifs are partially
complementary and predicted to form a stem-loop structure via base pairing, which may regulate mRNA stability or translation efficiency (Javelle et al., 2011; Klein-Cosson et al., 2015). The two characteristic motifs of HD-ZIP IV genes are also present in the 3′-UTR of URL1. We provide several lines of evidence to support its role in fine-tuning HD-ZIP IV mRNA stability. First, we found the potential cleavage sites within or surrounding the two conserved motifs (Figure 3E). Second, the nuclear run-on assay proved that nascent transcript level was not altered in the URL1 mutant, excluding the possibility that the C679T mutation affects transcription (Figure 3E). Third, the mutated URL1 (C679T) mRNA degraded slower than the WT URL1 mRNA after inhibiting transcription using cordycepin, suggesting that the mutated URL1 mRNA has increased stability (Figure 3F).

Although we have revealed the essential role of the URL1 3′-UTR in fine-tuning mRNA stability, the underlying molecular mechanism awaits further study. In mammals, the stem-loop structure in the 3′-UTR has been characterized as an mRNA destabilizing element (Paschoud et al., 2006; Mullen and Marzluff, 2008; Leppek et al., 2013; Chang et al., 2016). Stem-loop–binding proteins (SLBPs) such as SLBP, Roquin, and Roquin2 promote degradation of the histone and tumor necrosis factor-α mRNA after binding to the stem-loop structure (Mullen and Marzluff, 2008; Leppek et al., 2013). Hence, we speculate that the stem-loop structure formed in the 3′-UTR of URL1 may also serve as a binding site for certain proteins that promote mRNA degradation. The WT URL1 mRNA level was fine-tuned due to the stem-loop structure formed between the conserved CNS1 and CNS2 motifs in the 3′-UTR. In the URL1 mutant, the C679T substitution in the CNS2 motif reduced the complementarity between CNS1 and CNS2, making the stem-loop structure unstable. This may explain why WT URL1 mRNA did not over-accumulate in B197 transgenic rice plants, which did not show the adaxial leaf-rolling phenotype (Supplemental Figure S6, B and C). Our study provides an alternative mechanism for expression regulation of HD-ZIP IV family genes besides the translational repression proposed by a previous study on the maize OCL1 gene (Klein-Cosson et al., 2015).

URL1 serves as a transcriptional repressor by recruiting corepressor TPL2 via its EAR motif

Most EAR motif-containing proteins negatively regulate the expression of their target genes (Kagale et al., 2010). In agreement, we provided several lines of evidence to prove that URL1 serves as a transcriptional repressor in rice and that the EAR motif is required for recruiting the corepressor TPL2 (Figure 5). More importantly, the interaction between URL1 and TPL2 is required for the proper function of URL1 in regulating leaf rolling. Knockout of TPL2 in the Url1 mutant background significantly reduced adaxial rolling of the Url1 leaf, partly by increasing the expression of ACL1, the target gene of URL1 (Figure 6; Supplemental Figure S10). However, the leaf-rolling phenotype was not completely suppressed in the tpl2/Url1 mutants (Figure 6, B and C), although the expression level of ACL1 in the tpl2/Url1 mutants was significantly higher than that in WT (Supplemental Figure S10), suggesting that besides ACL1, TPL2 might target other genes in the modulation of leaf rolling. TPL and TPL-RELATED (TPR) proteins generally interact with different transcription factors and mediate transcriptional repression in a variety of development processes (Liu and Karmarkar, 2008; Szemenyei et al., 2008; Pauwels et al., 2010; Zhu et al., 2010; Causer et al., 2012; Wang et al., 2013; Hao et al., 2014). Consistently, the TPL2 knockout plants in the Url1 mutant background exhibited pleiotropic phenotypes. Spikelets on the upper part of the panicle showed enlarged sterile lemma (Supplemental Figure S10, C and D) and reduced stamen number (Supplemental Figure S10, G–I). Spikelets on the lower part of the panicle were pale white and degenerated (Supplemental Figure S10C).

In Arabidopsis, several HD-ZIP IV family members such as ATML1, PDF2, and GL2 directly and negatively regulate the expression of target genes (San-Bento et al., 2014; Lin et al., 2015). PDF2, GL2, and five other members of the Arabidopsis HD-ZIP IV family were predicted to harbor an EAR motif (Kagale et al., 2010). However, transcriptional repression activity has not been experimentally verified for any of them. Our work establishes transcriptional repression activity for HD-ZIP IV family members, which are supposed to act as transcriptional activators (Depege-Fargeix et al., 2011; Peterson et al., 2013).

URL1 cooperates with ROC5 in modulating leaf rolling

Interestingly, URL1 interacts with ROC5 in yeast and in planta (Figure 7); both of these proteins negatively regulate bulliform cell development. Loss-of-function mutation of either ROC5 (Zou et al., 2011) or URL1 (Supplemental Figure S4) resulted in increased number and size of bulliform cells and abaxially rolled leaves, suggesting that they are not functionally equivalent. The adaxially rolled-leaf phenotype resulting from the gain-of-function mutation of URL1 was alleviated when ROC5 was knocked out (Figure 8; Supplemental Figure S8). In addition, instead of the semi-rolled leaf of the Url1/+ hetrozygote (Figure 8), the Url1/+ oul1 mutant showed a flat-leaf phenotype comparable to WT, suggesting that ROC5 is required for the proper function of URL1. However, the Url1 oul1 double mutant still exhibited adaxially semi-rolled leaves. These genetic data suggest that ROC5 is required but not essential for URL1 function in modulating leaf rolling. Considering that the URL1/ROC5 heterodimer exhibited higher interaction strength than the URL1/URL1 or ROC5/ROC5 homodimers (Figure 7, A, B, D, and E), we speculate that URL1 and ROC5 preferentially function as a heterodimer in modulating leaf rolling. Meanwhile, URL1 can form a homodimer in the Url1 background, which
accumulated more URL1 transcripts than WT, and regulate leaf rolling.

It is noteworthy that ROC5 also has a canonical EAR motif at the N-terminus (Figure 5A). Both yeast two-hybrid assay and luciferase complementation assay in *N. benthamiana* leaves confirmed that ROC5 can also interact with TPL2 in vitro and in vivo (Supplemental Figure S11). Therefore, URL1, ROC5, and TPL2 may form a transcriptional repression complex. HD-Zip IV family proteins have been shown to recognize the L1 box motif (5'-TAAATGT-3'; Ohashi et al., 2003; Nakamura et al., 2006). Our data show that URL1 can bind with the L1 boxes in the promoter of the *ACL1* gene and suppress its expression (Figure 9), thus negatively regulating a positive regulator of bulliform cell development.

Collectively, our study integrates several genes controlling bulliform cell development into a regulatory network as shown in Figure 11. A transcriptional repression complex composed of URL1, ROC5, and the transcriptional corepressor TPL2 suppresses expression of the *ACL1* gene and modulates leaf rolling consequently. In WT, the stem-loop structure mediated by the conserved CNS1 and CNS2 motifs in the 3'-UTR triggers degradation of a portion of the URL1 mRNA. The modest amount of URL1 protein produced from the remaining URL1 transcript interacts with ROC5 and recruits corepressor protein TPL2, which inhibits the expression of target genes and maintains flat leaves. In the *Url1* mutant, a C-to-T substitution in the second motif CNS2 of 3'-UTR increased the mRNA stability and abundance. The abundant URL1 protein would form a homodimer as well as the URL1/ROC5 heterodimer to further repress the URL1 target genes, leading to a reduction in the number and size of bulliform cells to result in adaxially rolled leaves.

**Figure 11** A working model for the URL1 gene in the regulation of adaxial rolling of leaf. In WT, a part of the URL1 transcripts were cleaved and degraded due to the stem-loop structure mediated by the conserved CNS1 and CNS2 motifs in the 3'-UTR. The URL1 protein produced from the remaining transcript and ROC5 form a heterodimer and interact with the transcriptional corepressor TPL2 through the EAR motif to inhibit the expression of URL1 target genes such as *ACL1* that regulates bulliform cell development, maintaining flat leaves. In the *Url1* mutant, a C-to-T substitution in the second motif CNS2 of 3'-UTR increased the mRNA stability and abundance. The abundant URL1 protein would form a homodimer as well as the URL1/ROC5 heterodimer to further repress the URL1 target genes, leading to a reduction in the number and size of bulliform cells.

**Materials and methods**

**Plant material and growth conditions**

Rice plants were grown in the paddy fields of the Chinese Academy of Agricultural Sciences (Beijing, China) under natural growing conditions. Seedlings for protoplast isolation, ChIP, Co-IP, etc. were grown in a phytotron with 16-h light (30°C) and 8-h dark (22°C). The LRI of flag leaves at maturity was calculated by the following formula: LRI (%) = (Lw−Ln)/Lw*100. Ln and Lw indicated the distance of leaf blade...
map of the RNAi hairpin constructs for URL1 and TPL2

Histology and microscopy observations
For paraffin sectional analysis, the middle part of each leaf was taken at the time of anthesis and fixed in FAA solution followed by dehydration using a graded ethanol series. Sections 5- to 6-μm thick were cut and photographed as described previously (Fang et al., 2018). The longitudinal arrangement of bulliform cells were analyzed using mature leaf blade (12th leaf) as described previously (Zou et al., 2022). For semithin sectional analysis, the mature leaves were cut into small pieces and fixed in 2.5% (v/v) glutaraldehyde overnight at room temperature. Samples were subsequently washed three times in phosphate buffer (pH 7.2), dehydrated in ethanol, and embedded in Spurr resin as described previously (Zhao et al., 2012). The embedded samples were cut into 1-μm sections using an RMC MT-7 ultramicrotome (Reichert-Jung, Depew, NY, USA). Semithin sections were stained with 0.25% (w/v) TBO and photographed under an Olympus BX53 microscope. The ImageJ (http://rsb.info.nih.gov/ij/) was used to measure the epidermal cell area at the abaxial side of the leaf.

Map-based cloning of URL1
To map the URL1 locus, 309 plants showing an extremely rolled leaf phenotype in the F2 population derived from a cross betweenUrl1 and Dular and newly developed InDel markers were used (Supplemental Table S1). To identify the mutation site, genomic DNA fragments of candidate genes were sequenced using the web-based tool CRISPR-P (Lei et al., 2016). To construct the URL1 promoter-driven GUS reporter gene, a 2,566-bp fragment upstream of the URL1 start codon was inserted in pCAMBIA1305.1. To construct the rice Activation Tag91 promoter driven ACL1 over-expression vector, the CDS plus the 5′- and 3′-UTR of ACL1 gene was ligated into pCAMBIA1305.1-APFH (Supplemental Figure S12). All the primers used in vector construction are listed in Supplemental Table S2.

Modified RNA ligase-mediated 5′-RACE
Modified RLM 5′-RACE was performed using the FirstChoice RLM-RACE kit (Invitrogen). Poly A+ mRNA was purified from 1 mg total RNA extracted from 2-week-old seedlings using Oligotex (Qiagen). The purified mRNA (without calf intestine alkaline phosphatase treatment) was ligated to 5′-RACE RNA adaptors for reverse transcription. The cDNA samples were amplified by nested PCR using the gene-specific primers URL1(UTR)R1 (5′-TTTTTTTTCAAAAAATTGTTTTAAG-3′) and URL1(UTR)R2 (5′-CCATTATTACCGA AATGAATGTTC-3′) for the first and second round PCR, respectively. The second round PCR products were cloned in pEASY-Blunt Simple Cloning Vectors (Transgen Biotech) and sequenced.

Nuclear run-on assay
Nuclear run-on assay was performed as described previously with slight modifications (Roberts et al., 2015). Briefly, 6 g of 7-d-old Url1 mutant and WT seedlings were harvested for isolating nuclei by centrifuging using Percoll density gradients (Folta and Kaufman, 2006). The nuclei layer was gently resuspended in 300-μL storage buffer (50 mM Tris–HCl pH 7.8, 10 mM 2-mercaptoethanol, 20% (v/v) glycerol, 5 mM MgCl2, and 0.44 M sucrose), snap-frozen in liquid nitrogen, and then stored at −80 °C until use. Run-on reaction was based on the incorporation of biotin-16-uridine-5′-triphosphate (biotin-16-UTP) into nascent transcripts (Patrone et al., 2000). Approximately 106 nuclei in 50-μL storage buffer were incubated with 20 U RNAsin (Promega) for 10 min at 30°C to be pre-warmed. Then 10 μL 10-fold pre-warmed transcription buffer (working concentration: 25 mM Tris–HCl pH 7.8, 37.5 mM NH4Cl, 5 mM MgCl2, 5% (v/v) glycerol, 1 mM each of ATP, GTP, and CTP) and 1 mM biotin-16-UTP (Roche Diagnostics) was added to each sample and the transcription reaction continued for 45 min at 30°C. The reaction was stopped by placing the samples on ice. Immediately, total RNA was isolated with TRIzol reagent (Invitrogen) and treated with RNase free DNase I (Roche). Total RNA was then incubated with streptavidin magnetic beads (NEB) to immunoprecipitate biotinylated RNAs and used for reverse-transcription with random decamers to

Margins at natural state and the distance of leaf blade margins at unfolding state, respectively (Shi et al., 2007).

Constructs for transgenic plants
To construct the genomic DNA complementation vector, the 5,956-bp URL1 genomic DNA sequence (including 1,940-bp upstream of ATG, and 946-bp downstream of TGA) was amplified from the WT and Url1 mutant leaf cDNA and ligated into the binary vector pCAMBIA1305.1-APFHN (Supplemental Figure S12). To construct the URL1 over-expression vector, the URL1 coding sequence (CDS) plus the 5′-UTR was amplified from the WT and Url1 mutant leaf cDNA and ligated into the binary vector pCAMBIA1305.1

BP reaction with URL1attB-flanked fragment and pDONR/Zeo vector was performed to generate the URL1 entry clone. We then performed LR reaction with URL1 entry clone and the plant binary vector LP041 nEAR-hyg-asRED or LP042 nVP64-hyg-asRED (Zhao et al., 2015). To construct the DEX-inducible expression vector of URL1, the URL1 CDS plus 3′-UTR was ligated into vector pINDEX3 (Ouwerkerk et al., 2001). To construct the URL1 promoter-driven GUS reporter gene, a 2,566-bp fragment upstream of the URL1 start codon was inserted in pCAMBIA1305.1. To construct the rice Actin9 promoter driven ACL1 over-expression vector, the CDS plus the 5′- and 3′-UTR of ACL1 gene was ligated into pCAMBIA1305.1-APFH (Supplemental Figure S12). All the primers used in vector construction are listed in Supplemental Table S2.

Modified RNA ligase-mediated 5′-RACE
Modified RLM 5′-RACE was performed using the FirstChoice RLM-RACE kit (Invitrogen). Poly A+ mRNA was purified from 1 mg total RNA extracted from 2-week-old seedlings using Oligotex (Qiagen). The purified mRNA (without calf intestine alkaline phosphatase treatment) was ligated to 5′-RACE RNA adaptors for reverse transcription. The cDNA samples were amplified by nested PCR using the gene-specific primers URL1(UTR)R1 (5′-TTTTTTTTCAAAAAATTGTTTTAAG-3′) and URL1(UTR)R2 (5′-CCATTATTACCGA AATGAATGTTC-3′) for the first and second round PCR, respectively. The second round PCR products were cloned in pEASY-Blunt Simple Cloning Vectors (Transgen Biotech) and sequenced.
generate cDNA (Takara). Nascent URL1 mRNA levels were measured via RT-qPCR with primers listed in Supplemental Table S3 and the rice Actin gene was used as an internal control.

mRNA decay assay
mRNA decay assay was performed as described previously (Niu et al., 2019). A 7-d-old Url1 mutant and WT seedlings were immersed in incubation buffer (1 mM PIPES (1,4-Piperazinediethanesulfonic acid), pH 6.25, 1 mM trisodium citrate, 1 mM KCl, 15 mM sucrose) for 30 min. Cordycepin (Sigma C-3394) was supplemented to a final concentration of 200 μg mL⁻¹ and samples were vacuum infiltrated for 5 min. Shoot bases were collected at different time points to extract RNA. RT-qPCR analysis was conducted using ubiquitin as an internal control. Five biological replicates were used at each time point.

Reverse transcription quantitative PCR
Total RNA was extracted from different tissues or protoplasts using TRIzol reagent (Invitrogen). First-strand cDNA was synthesized from 2 μg of total RNA using a SuperScript II kit (TaKaRa). RT-qPCR was performed using the ABI prism 7500 real-time PCR System with SYBR Premix Ex Taq kit (TaKaRa). RT-qPCR was performed using the ABI prism 7000 real-time PCR System with SYBR Premix Ex Taq kit (TaKaRa). The rice ubiquitin gene was used as an internal control. The relative gene expression level was calculated using the 2⁻ΔΔCT method (Livak and Schmittgen, 2001). Primers are listed in Supplemental Table S3.

Histochemical GUS assay
Histochemical assay of the transgenic rice plants harboring the URL1pro-GUS construct was performed as described previously (Jefferson et al., 1987).

In situ hybridization
Shoot bases of 7-d-old seedlings from WT and the Url1 mutant were fixed in 4% (w/v) paraformaldehyde at 4°C overnight, followed by a dehydration series and infiltration, and embedded in paraffin (Paraplast Plus, Sigma). The tissues were sliced into about 8-μm sections with a microtome (Leica RM2145), and the sections were mounted on RNase-free glass slides. A 292-bp fragment within the 3′-UTR region (Supplemental Table S3) was used as the template to generate sense and antisense RNA probes that were labeled using a DIG Northern Starter Kit (catalog no. 2039672; Roche). RNA in situ hybridization with probes was performed on sections.

Subcellular localization
The URL1 CDS was ligated in the transient expression vector pAN580 having the GFP encoding gene and transformed into rice protoplasts (Sheng et al., 2014). GFP signals were observed using a confocal laser scanning microscope at 488 nm (Leica SP2).

Luciferase transient expression assays in rice protoplasts
The constructs used to examine effect of the URL1 3′-UTR on gene expression were prepared as follows. First, the empty reporter construct pAN121 was developed from the transient expression vector pAN580 (Sheng et al., 2014). The LUC-coding sequence was amplified from the plasmid Pro35S-GAL4UAS-LUC (Lin et al., 2013) and ligated into pAN580 replacing the GFP gene. Then, the URL1 3′-UTR was amplified from the WT and Url1 mutant genomic DNA and inserted in the pAN121 vector. The internal control vector p35S-RLuc was developed from pAN580 by replacing the GFP gene with the Renilla LUC gene. The fusion constructs used to examine the transcriptional repression activity of the URL1 protein were made as follows. The reporter plasmid Pro35S-GAL4UAS-LUC was adopted from a previous study (Lin et al., 2013). The empty effector plasmids Pro35S-GAL4BD and Pro35S-GAL4BD:VP16 were constructed previously (Peng et al., 2017). To create the effector construct without the VP16 motif, the coding sequences of URL1 was inserted into Pro35S-GAL4BD:VP16 vector using primers URL1BBF/R. To create the effector construct containing the VP16 motif, the coding sequences of URL1 was ligated into Pro35S-GAL4BD:VP16 vector using primers URL1BBF/R. Protoplasts were isolated from rice seedlings and transformed as described previously (Yoo et al., 2007). In the assay for URL1 transcriptional repression activity, 5 μg of reporter plasmid, 4 μg of effector plasmid, and 1 μg of internal control plasmid pAN508 (Pro35S-GUS) were used for each transformation event. The firefly luciferase activity was measured on a Centro XS³ LB 960 High Sensitivity Microplate Luminometer (Berthold Technologies). The GUS activity of the same sample was measured using the GUS extraction buffer on fluorescence spectrophotometer F-4600 (Hitachi). The relative activity of the different reporter constructs is expressed as the LUC/GUS ratio. Three biological replicates were used. In the assay for the URL1 3′-UTR regulatory roles on gene expression, 5 μg of reporter plasmid and 1 μg of internal control plasmid (p35S-Renilla LUC) was added to each transformation. The firefly and Renilla luciferase activities were monitored using the Dual-Luciferase Reporter Assay System (Promega). The relative activity of different reporter constructs was calculated as the LUC/RLuc ratio.

Yeast two-hybrid analysis
N-termius of TPL2 was cloned into bait vector pGBK7. Full-length coding sequence of URL1, URL1mEAR, and ROC5 were respectively cloned into prey vector pGAD7. To test the dimerization between URL1 and ROC5, the full-length CDS of URL1 and ROC5 were cloned into pGBK7 or pGAD7. Different combinations of prey and bait vectors were co-transformed into yeast stain AH109 as described previously (Zhang et al., 2017).
Luciferase complementation imaging assay

The full-length URL1, URL1mEAR, ROC5, and N-terminus of TPL2 were fused with the N- or C-terminal parts of the reporter gene LUC to generate CLUC-URL1, CLUC-URL1mEAR, CLUC-ROC5, and NLUC-TPL2. All the constructs were transformed into Agrobacterium tumefaciens strain EHA105. The bacteria with OD₆₀₀ = 0.5 were collected and resuspended using activity buffer (10 mM Methylmethanesulfonic acid (MES), pH 5.7, 10 mM MgCl₂, 150 mM acetylsyringone). An equal volume of A. tumefaciens harboring different CLUC and NLUC construct pairs were mixed to a final concentration of OD₆₀₀ = 1.0. Four or six different combinations of the ROC5 promoter (Supplemental Table S4) were labeled at the 3′-end with biotin and annealed to form the double-stranded DNA probe. The labeled probes were used as competitors in the competing assays. Labeled probes and their co-immunoprecipitates were detected in an automatic chemiluminescence image analysis system (Tanon 5200). Quantitative analysis was performed using IndiGo software (Berthold Technologies). The ChIP assay was performed as previously described (Supplemental Figure S1). The enrichment of particular regions in the ACL1 promoter (Supplemental Table S4) was quantified by qPCR as described previously.

DEX treatment

Calli from WT and the T₃ seeds of pINDEX3-URL1 transgenic rice plants were induced. For the DEX or mock treatment, the transgenic calli were transferred to solid MS medium plates containing 30 μM DEX (Sigma, D1756) or equal volumes of DMSO. Samples were harvested at 96, 120, and 130 h after treatment and used for RNA extraction. Three biological replicates were used at each time point for analysis, and it was repeated three times.

Accession numbers

Sequence data from this article can be found in the GenBank databases or the Rice Genome Annotation Project website (http://rice.plantbiology.msu.edu/) under the following accession numbers: URL1 genomic DNA, MH822134; URL1 cDNA, MH822135; ROC5, LOC_Os02g45250; TPL2, LOC_Os08g06480; ACL1, LOC_Os04g33860.

Supplemental data

The following supplemental materials are available.

Supplemental Figure S1. Cross section of the wild type (WT) and Url1 mutant mature leaf.

Supplemental Figure S2. Sequence alignment of OsURL1, ZmOCL1, and AtGL2.

Supplemental Figure S3. Phylogenetic analysis of rice, maize, and Arabidopsis HD-ZIP IV family members.

Supplemental Figure S4. Targeted mutagenesis of the URL1 gene using CRISPR-Cas9 technique.

Supplemental Figure S5. The secondary structure in the 3′-UTR of URL1 predicted by the RNAfold software.

Supplemental Figure S6. Characterization of the Url1 and URL1 cDNA overexpression lines.

Supplemental Figure S7. Tissue-specific expression of URL1.

Supplemental Figure S8. Characterization of the ROC5 mutant created via CRISPR-Cas9 in the Url1 mutant background.

Supplemental Figure S9. Effects of DEX on the expression of the URL1 and its target gene ACL1 in WT and DEX-treated calli.

Supplemental Figure S10. Phenotypes of abnormal spikelet in the tpl2/Url1 mutants generated via CRISPR-Cas9.

Supplemental Figure S11. ROC5 interacts with the N-Terminus of TPL2.

Supplemental Figure S12. Schematic representation of the plant expression vectors pCAMBIA1305.1-APFHN and pCAMBIA1305.1-APFHC.

Supplemental Table S1. Primers of INDEL markers used in map-based cloning.
Supplemental Table S2. List of primers used in vector construction.
Supplemental Table S3. List of primers used in RT-qPCR and in situ hybridization.
Supplemental Table S4. List of primers used in EMSA and ChIP.

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Conflict of interest statement
The authors have no conflicts of interest to declare.

References
Abe M, Katsumata H, Komeda Y, Takahashi T (2003) Regulation of shoot epidermal cell differentiation by a pair of homeodomain proteins in Arabidopsis. Development 130: 635–643
Aguilera V, Berenguer M, Palau A, Rubin A, Aguas M, Benlloch S, Prieto M (2007) Response to pegylated interferon-ribavirin in patients with recurrent hepatitis C in patients undergoing liver transplantation for mixed (alcohol-HCV) cirrhosis. J Hepatol 47: 196–202
Burgess D, Freeling M (2014) The most deeply conserved noncoding sequences in plants serve similar functions to those in vertebrates despite large differences in evolutionary rates. Plant Cell 26: 946–961
Causer B, Ashworth M, Guo W, Davies B (2012) The TOPLESS interactor: a framework for gene repression in Arabidopsis. Plant Physiol 158: 423–438
Chang SF, Li HC, Huang YP, Tasi WJ, Chou YY, Lu SC (2016) SB203580 increases G-CSF production via a stem-loop destabilizing element in the 3ʼ untranslated region of macrophages independently of its effect on p38 MAPK activity. J Biomed Sci 23: 3
Depepe-Fargeix N, Javelle M, Chambrier P, Frangne N, Gerentes D, Perez P, Rogowsky PM, Vernoud V (2011) Functional characterization of the HD-ZIP IV transcription factor OCL1 from maize. J Exp Bot 62: 293–305
Dhindsa RS, Plumb Dhinda P, Thorpe TA (1981) Leaf senescence: correlated with increased levels of membrane permeability and lipid peroxidation, and decreased levels of superoxide dismutase and catalase. J Exp Bot 32: 93–101
Dudley M, Poethig RS (1993) The heterochronic Teopod1 and Teopod2 mutations of maize are expressed non-cell-autonomously. Genetics 133: 389–399
Elhiti M, Stasolla C (2009) Structure and function of homodomain-leucine zipper (HD-Zip) proteins. Plant Signal Behav 4: 86–88
Fang J, Yuan S, Li C, Jiang D, Zhao L, Peng L, Zhao J, Zhang W, Li X (2018) Reduction of ATPase activity in the rice kinesin protein Stemless Dwarf 1 inhibits cell division and organ development. Plant J 96: 620–634
Fang L, Zhao F, Cong Y, Sang X, Du Q, Wang D, Li Y, Ling Y, Yang Z, He G (2012) Rolling-leaf14 is a 2OG-Fe (II) oxygenase family protein that modulates rice leaf rolling by affecting secondary cell wall formation in leaves. Plant Biotechnol J 10: 524–532
Folta KM, Kaufman LS (2006) Isolation of Arabidopsis nuclei and measurement of gene transcription rates using nuclear run-on assays. Nat Protoc 1: 3094–3100
Gendrel AV, Lippman Z, Martienssen R, Colot V (2005) Profiling histone modification patterns in plants using genomic tiling microarrays. Nat Methods 2: 213–218
Gruber AR, Lorenz R, Bernhart SH, Neubock R, Hofacker IL (2008) The Vienna RNA websuite. Nucleic Acids Res 36: W70–W74
Hao Y, Wang X, Li X, Bassa C, Mila I, Audran C, Maza E, Li Z, Bouzyan M, van der Rest B, Zouine M (2014) Genome-wide identification, phylogenetic analysis, expression profiling, and protein–protein interaction properties of TOPESS gene family members in tomato. J Exp Bot 65: 1013–1023
Hernandez ML, Passas HJ, Smith LG (1999) Clonal analysis of epidermal patterning during maize leaf development. Dev Biol 216: 646–658
Hibara K, Obara M, Hayashida E, Abe M, Ishimaru T, Satoh H, Itoh J, Nagato Y (2009) The ADAXIALIZED LEAF1 gene functions in leaf and embryonic pattern formation in rice. Dev Biol 334: 345–354
Holden KL, Harris E (2004) Enhancement of dengue virus translation: role of the 3ʼ untranslated region and the terminal 3ʼ stem-loop domain. Virology 329: 119–133
Hori K, Watanabe Y (2008) Chapter 8. In vivo analysis of plant nonsense-mediated mRNA decay. Methods Enzymol 449: 165–176
Ito M, Sentoku N, Nishimura A, Honk SK, Sato Y, Matsuoka MJBe (2003) Roles of Rice GL2-type Homeobox Genes in Epidermis Differentiation. Breeding Sci 53: 245–253
Itoh J, Nonomura K, Ikeda K, Yamaki S, Inukai Y, Yamagishi H, Kitano H, Nagato Y (2005) Rice plant development: from zygote to spikelet. Plant Cell Physiol 46: 23–47
Javelle M, Klein-Cosson C, Vernoud V, Boltz V, Maher C, Timmermans M, Depepe-Fargeix N, Rogowsky PM (2011) Genome-wide characterization of the HD-ZIP IV transcription factor family in maize: preferential expression in the epidemis. Plant Physiol 157: 790–803
Jefferson RA, Kavanagh TA, Bevan MW (1987) GUS fusions: beta-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. EMBO J 6: 3901–3907
Jiao Y, Wang Y, Xue D, Wang J, Yan M, Liu G, Dong G, Zeng D, Lu Z, Zhu X, Qian Q, Li J (2010) Regulation of OssPL14 by OsMIR156 defines ideal plant architecture in rice. Nat Genet 42: 544–544
Kadioglu A, Terzi R (2007) A dehydration avoidance mechanism: leaf rolling. Botanical Review 73: 290–302
Kagale S, Links MG, Rozwadowski K (2010) Genome-wide analysis of ethylene-responsive element binding factor-associated amphipathic repression motif-containing transcriptional regulators in Arabidopsis. Plant Physiol 152: 1109–1134
Klein-Cosson C, Chambrier P, Rogowsky PM, Vernoud V (2015) Regulation of a maize HD-ZIP IV transcription factor by a non-conventional RDR2-dependent small RNA. Plant J 81: 747–758
Lang Y, Zhang Z, Gu X, Yang J, Zhu Q (2004) Physiological and ecological effects of cramped leaf character in rice (Oryza sativa L.) II. Photosynthetic character, dry mass production and yield forming. Acta Agronomica Sinica 30: 883–887
Lei Y, Lu L, Liu HY, Li S, Xing F, Chen LL (2014) CRISPR-P: a web tool for synthetic single-guide RNA design of CRISPR-system in plants. Mol Plant 7: 1494–1496
Leppik K, Schott J, Reiter S, Poetz F, Hammond MG, Stoecklin G (2013) Roquin promotes constitutive mRNA decay via a conserved class of stem-loop recognition motifs. Cell 153: 869–881
Li H, Jiang L, Youn JH, Sun W, Cheng Z, Jin T, Ma X, Guo X, Wang J, Zhang X et al. (2013) A comprehensive genetic study reveals a crucial role of CYP90D2/D2 in regulating plant architecture in rice (Oryza sativa). New Phytol 200: 1076–1088

Li L, Shi ZY, Li L, Shen GZ, Wang XQ, An LS, Zhang JL (2010) Overexpression of ACL1 (abaxially curled leaf 1) increased Bulliform cells and induced Abaxial curling of leaf blades in rice. Mol Plant 3: 807–817

Lin H, Niu L, McHale NA, Ohme-Takagi M, Mysore KS, Tadege M (2013) Evolutionarily conserved repressive activity of WOX proteins mediates leaf blade outgrowth and floral organ development in plants. Proc Natl Acad Sci USA 110: 366–371

Lin Q, Ohashi Y, Kato M, Tsuge T, Gu H, Qu LJ, Aoyama T (2015) GLABRA2 directly suppresses basic helix-loop-helix transcription factor genes with diverse functions in root hair development. Plant Cell 27: 2894–2906

Liu Z, Karmarkar V (2008) Groucho/Tup1 family co-repressors in plant development. Trends Plant Sci 13: 137–144

Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 25: 402–408

Ma X, Zhang Q, Zhu Q, Liu W, Chen Y, Qiu R, Wang B, Yang Z, Li H, Lin Y et al. (2015) A robust CRISPR/Cas9 system for convenient, high-efficiency multiplex genome editing in monocot and dicot plants. Mol Plant 8: 1274–1284

Moon J, Hake S (2011) How a leaf gets its shape. Curr Opin Plant Biol 14: 24–30

Mullen TE, Marzluff WF (2008) Degradation of histone mRNA requires oligouridylation followed by decapping and simultaneous degradation of the mRNA both 5′ to 3′ and 3′ to 5′. Genes Dev 22: 50–65

Nakamura M, Katsumata H, Abe M, Yabe N, Komedya Y, Yamamoto KT, Takahashi T (2006) Characterization of the class IV homeodomain-Leucine zipper gene family in Arabidopsis. Plant Physiol 141: 1363–1375

Niu XM, Xu YC, Li ZW, Bian YT, Hou XH, Chen JF, Zou YP, Jiang J, Wu Q, Ge S et al. (2019) Transposable elements drive rapid phenotypic variation in Capsella rubella. Science 363: 761–766

Ohashi Y, Oka A, Rodrigues-Pousada R, Possenti M, Ruberti I, O’Toole JC, Cruz RT, Singh TNPSL (1979) Leaf rolling and transpiration. Plant Science Letters 16: 111–114

Ohashi Y, Oka A, Rodrigues-Pousada R, Possenti M, Ruberti I, Morelli G, Aoyama T (2003) Modulation of phospholipid signaling by GLABRA2 in root-hair pattern formation. Science 300: 1427–1430

Ouwerkerk PB, de Kam RJ, Hoge JH, Meijer AH, Ijdo JW, Kornberg RD, Reasons AM (2000) A versatile cell system for transient gene expression analysis. Nat Protoc 23: 1565–1572

Paschoud S, Dogar AM, Kuntz C, Grisoni-Neupert B, Richman L, Kuhn LC (2006) Destabilization of interleukin-6 mRNA requires a putative RNA stem-loop structure, an AU-rich element, and the RNA-binding protein AUFI. Mol Cell Biol 26: 8228–8241

Patrone G, Puppo F, Cusano R, Scaranari M, Ceccherini I, Puliti A, Ravazzolo R (2000) Nuclear run-on assay using biotin labeling. Magnetic bead capture and analysis by fluorescence-based RT-PCR. Biotechniques 29: 1012–1014

Pauwels L, Barbero GF, Geerinck J, Tilleman S, Grunewald W, Perez AC, Chico JM, Bossche RV, Sewell J, Gil E et al. (2010) NJINJA connects the co-repressor TOPLESS to jasmonate signalling. Nature 464: 788–791

Peng P, Liu L, Fang J, Zhao J, Yuan S, Li X (2017) The rice TRIANGULAR HULL1 protein acts as a transcriptional repressor in regulating lateral development of spikelet. Sci Rep 7: 13712

Peterson KM, Shyu C, Burr CA, Horst RJ, Kanaoka MM, Omae M, Sato Y, Torii KU (2013) Arabidopsis homeodomain-leucine zipper IV proteins promote stomatal development and ectopically induce stomata beyond the epidermis. Development 140: 1924–1935

Robert TC, Hart JR, Kaikonen MU, Weinberg MS, Vogt PK, Morris KV (2015) Quantification of nascent transcription by bromouridine immunocapture nuclear run-on RT-qPCR. Nat Protoc 10: 1198–1211

Ross J (1995) mRNA stability in mammalian cells. Microbiol Rev 59: 423–450

Ruberti I, Sessa G, Lucchetti S, Morelli G (1991) A novel class of plant proteins containing a homeodomain with a closely linked leucine zipper motif. EMBO J 10: 1787–1791

Sadowski I, Ma J, Triebenzer P, Ptashne M (1988) GAL4-VP16 is an unusually potent transcriptional activator. Nature 335: 563–564

San-Bento R, Farbot E, Galletti R, Creff A, Ingram G (2014) Epidermal identity is maintained by cell-cell communication via a universally active feedback loop in Arabidopsis thaliana. Plant J 77: 46–58

Sheng P, Tan J, Jin M, Wu F, Zhou K, Ma W, Heng Y, Wang J, Guo X, Zhang X et al. (2014) Albino midrib 1, encoding a putative potassium efflux antiporter, affects chloroplast development and drought tolerance in rice. Plant Cell Rep 33: 1581–1594

Shi Z, Wang J, Wan X, Shen G, Wang X, Zhang J (2007) Over-expression of rice OsAG07 gene induces upward curling of the leaf blade that enhanced erect-leaf habit. Planta 226: 99–108

Simone LE, Keene JD (2013) Mechanisms coordinating ELAV/Hu mRNA regulons. Curr Opin Genet Dev 23: 35–43

Szemenyei H, Hannon M, Long JA (2008) TOPLESS mediates auxin-dependent transcriptional repression during Arabidopsis embryogenesis. Science 319: 1384–1386

Tavares R, Aubourg S, Lecharny A, Kreis M (2000) Organization and structural evolution of four multigene families in Arabidopsis thaliana: AtlCAD, AtlGT, AtMYST and AthD-GL2. Plant Mol Biol 42: 703–717

Vlasova IA, Bohjajen PR (2008) Posttranscriptional regulation of gene networks by GU-rich elements and CELF proteins. RNA Biol 5: 201–207

Wang L, Kim J, Somers DE (2013) Transcriptional corepressor TOPLESS complexes with pseudoresponse regulator proteins and histone deacetylases to regulate circadian transcription. Proc Natl Acad Sci USA 110: 761–766

Xiang JJ, Zhang GH, Qian Q, Xue HW (2012) Semi-rolled leaf1 encodes a putative glycosylphosphatidylinositol-anchored protein and modulates rice leaf rolling by regulating the formation of bulliform cells. Plant Physiol 159: 1488–1500

Xu Y, Wang Y, Long Q, Huang J, Wang Y, Zhou K, Zheng M, Sun J, Chen H, Chen S et al. (2014) Overexpression of OsZHD1, a zinc finger homeodomain class homeobox transcription factor, induces abaxially curled and drooping leaf in rice. Planta 239: 803–816

Yoo SD, Cho YH, Sheen J (2007) Arabidopsis mesophyll protoplasts: a versatile cell system for transient gene expression analysis. Nat Protoc 2: 1565–1572

Zalewski CS, Floyd SK, Furumizu C, Sakakibara K, Stevenson DW, Bowman JL (2013) Evolution of the class IV HD-zip gene family in streptophytes. Mol Biol Evol 30: 2347–2365

Zhang GH, Xu Q, Zhu XD, Qian Q, Xue HW (2009) SHALLOT-LIKE1 is an AKA NAD1 transcription factor that modulates rice leaf rolling by regulating leaf abaxial cell development. Plant Cell 21: 719–735, http://dx.doi.org/10.1105/tpc.108.061457

Zhang M, Zhao J, Li L, Gao Y, Zhao L, Patil SB, Fang J, Zhang W, Yang Y, Li M et al. (2017) The Arabidopsis U-box E3 ubiquitin ligase PUB30 negatively regulates salt tolerance by facilitating BR11 kinase inhibitor 1 (BK1I) degradation. Plant Cell Environ 40: 2831–2843

Zhang HZ, Wang DF, Speal B, Ma H (2002) The excess microsprotytes1 gene encodes a putative leucine-rich repeat receptor protein kinase that controls somatic and reproductive cell fates in the Arabidopsis anther. Genes Dev 16: 2031–2033

Zhao T, Liu J, Li HY, Lin JZ, Bian MD, Zhang CY, Zhang YX, Peng YC, Liu B, Lin C (2015) Using hybrid transcription factors to study gene function in rice. Sci China Life Sci 58: 1160–1162
Zhu Z, Xu F, Zhang Y, Cheng YT, Wiermer M, Li X, Zhang Y (2010) Arabidopsis resistance protein SNC1 activates immune responses through association with a transcriptional corepressor. Proc Natl Acad Sci USA 107: 13960–13965

Zou LP, Sun XH, Zhang ZG, Liu P, Wu JX, Tian CJ, Qiu JL, Lu TG (2011) Leaf rolling controlled by the homeodomain leucine zipper class IV gene Roc5 in rice. Plant Physiol 156: 1589–1602