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Ectopic expression of OsLFL1 in rice represses *Ehd1* by binding on its promoter

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Abstract

B3 domain was identified as a novel DNA-binding motif specific to higher plant species. The B3 proteins play important roles in plant development. In the mutant *W378*, the mutant gene coding *OsLFL1*, a putative B3 transcription factor gene, was ectopically expressed. In this study, it was found that the flowering promoting gene *Ehd1* and its putative downstream genes were all repressed by *OsLFL1*. Electrophoretic mobility shift assays (EMSA) and chromatin immunoprecipitation (ChIP) analyses suggest that OsLFL1 binds to the RY *cis*-elements (CATGCATG) in the promoter of the *Ehd1* gene. Thus, ectopically expressed OsLFL1 might repress *Ehd1* via binding directly to the RY *cis*-elements in its promoter.

Keywords: OsLFL1; RY *cis*-element; Ehd1; Expression; Rice

DNA-binding domains are one of the conserved motifs of transcription factors, binding specifically to the target DNA sequences [1]. A kind of B3 DNA-binding domain (pfam02362) specific for plants was identified recently. It was first reported in proteins Abscisic Acid-insensitive 3 (ABI3) from *Arabidopsis* and Viviparous 1 (VP1) from *Zea Mays*. Three basic domains, as B1, B2 and B3 and a single acidic domain, as A1, were characterized in those two proteins [2–4]. The B3 domain was identified as the DNA-binding motif. Up to now, more and more B3 domain containing proteins were identified in different plant species. These B3 proteins play important roles in signal transduction and plant development [2,3,5–10].

B3 proteins are commonly classified into three kinds of subfamilies based on their structures and functions: ABI3/VP1-likes (ABI3, VP1, LEC2, FUSCA3, and etc.), RAV-likes (RAV1, 2, and etc.), and auxin response factors (ARFs) families [11]. ABI3/VP1 bind specifically to two kinds of *cis*-elements: RY/Sph (CATGCATG) and G box (ACGT), but LEC2 and FUSCA3 from this subfamily bind only to RY elements. B3 proteins of this subfamily function mainly in plant seeds development and ABA signal transduction [2–4,6,10]. RAV-likes proteins are involved in bacterial disease resistance, drought and salt stress tolerance and their B3 domains bind to CACCTG elements [12,13]. B3 proteins of ARFs are found to bind to ARE (Auxin response elements, TGTCTC) [14]. ARFs might be regulated by microRNAs and participate in *Arabidopsis* flower and leaf development [15–17].

Despite that many characters were assigned to those B3 proteins in *Arabidopsis*, there are few reports on rice (*Oryza sativa L.*) B3 genes. In previous studied, a novel B3 DNA-binding domain containing protein OsLFL1 was isolated by T-DNA tagging from rice mutant *W378* generated in our lab (Supplementary Fig. S1) [18,19]. Genetic and transgenic analyses showed that *OsLFL1* (EF521182) was responsible for the late-flowering phenotype of *W378* (data

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not shown). Here we reported the expressional and functional analyses of *OsLFL1*. In mutant *W378*, *OsLFL1* was ectopically expressed and so that repressed the expression of a flowering promoting gene *Ehd1* [20]. Three copies of RY *cis*-elements (CATGCATG) were found in the promoter of *Ehd1*, electrophoretic mobility shift assays (EMSA) and chromatin immunoprecipitation (ChIP) analyses indicated that the OsLFL1 protein could interact with those RY *cis*-elements. Thus, OsLFL1 might regulate *Ehd1* gene expression by direct binding to its promoter.

Materials and method

Plant species and growth conditions. Rice Zh11 (*Oryza sativa L. subsp. japonica cv. Zhonghua No. 11*) was used as wild-type plants. Mutant *W378* was selected from rice Zh11 T-DNA insertion mutant line collections. Zh11 and mutant *W378* were grown in green houses (with 10, 12 or 14 h light a day, ~2, 700 μmol m^{-2} s^{-1}) for phenotype analysis.

RNA analysis. Total RNA was isolated from Zh11, *W378* and transgenic plants using the TRIzol reagent (Invitrogen). For RT-PCR analysis, 1 μg of total RNA was reverse transcribed using an oligo(dT) primer and M-MLV RTase (TOYOBO, Japan) according to the manufacturer's instructions. Primers for RT-PCR were listed in Supplementary Table S1. All final RT-PCR experiments were performed at least in three times.

Yeast one hybrid assay. To analyze OsLFL1 binding to the DNA fragments of *Ehd1* promoter, a yeast-one-hybrid system [21] constructed from the DupLEX-A two-hybrid system (Origene) was applied. Three DNA fragments of different length containing the wild-type RY repeats (CATGCATG) (A, B, and C, respectively) or the mutant RY repeats (CATAAATG) (Am, Bm, and Cm, respectively) of the *Ehd1* promoter was amplified by PCR. The DNA fragments were digested with XhoI and inserted before the mini-promoter of yeast plasmid p178 (URA3, 2 μm, Apr, mini-promoter-*lacZ*) to form the reporter plasmid DNA-p178. The *OsLFL1* cDNA (coding full-length protein, containing trans-activating domain) was then inserted into plasmid pEG202 to form the derivative OsLFL1-pEG202. OsLFL1-pEG202 and DNA-p178 were co-transformed into yeast cells and transformants were grown on SD medium in the absence of histidine and uracil, and X-gal plates for 2–3 days at 30 °C.

Electrophoretic mobility shift assays (EMSA) and competition experiments. The wild-type (A: +16—CATGCATG— −162, B: −163—CATGCATG— −582, C: −582— CATGCATG— −920) and mutant (Am: +16—CATAAATG— −162, Bm: −163—CATAAATG— −582, Cm: −582— CATAAATG— −920) DNA fragment in EMSAs were amplified by PCR with KOD plus (TOTOBO, Japan). The primer sequences used are available upon request. Those DNA fragments were labeled by γ-^{32}P using the T4 polynucleotide kinase in the presence of [γ-^{32}P]ATP. EMSAs were performed as described by Imaizumi [22] with minor changes: the OsLFL1 protein were prepared from *E. coli* strain BL21 (DE3) with buffer consisting of 50 mM NaH_2PO_4, 300 mM NaCl (pH 8.0) and used in EMSAs without further purification. Binding reactions were carried out in DNA-binding buffer containing 10 mM Tris–Cl, pH 8.0, 50 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, 10% glycerol and supplemented with 2.5 ng poly(dI-dC), 1 nM labeled DNA fragment and the indicated amount of cold competitor (50 and 100 nM). After addition of 100 ng total protein, the protein and DNA-binding mixtures were incubated at 25 °C for 20 min, and then subjected to electrophoresis on 6% acrylamide gels containing 1× TBE (22.5 mM Tris borate, pH 8.0, 0.25 mM EDTA). The gels were subsequently dried and exposed to X-ray films (Kodak).

Chromatin immunoprecipitation (ChIP) analysis. ChIP assays were performed mainly as described by Gendrel [23] and protocol of ChIP Assay Kit (Upstate, NY). Leaves of Zh11 and *W378* seedling (10 DAG, day after germination) were harvested and treated with 1% formaldehyde to cross-link the protein and DNA. The anti-OsLFL1 antibody was added to pull down the OsLFL1 and DNA complex (+anti-OsLFL1) in Zh11 and *W378* samples. The immunoprecipitated and purified DNA were then used as template in PCRs. Samples without anti-OsLFL1 were applied as negative control (−anti-OsLFL1) and input DNA of Zh11 and *W378* was used as positive control. In *Ehd1* gene promoter, positive primers (Ehd1P-1/2, 3/4, 5/6) were designed to span RY *cis*-elements (*Ehd1*-RY1, *Ehd1*-RY2, and *Ehd1*-RY3), respectively, and negative primers (Ehd1P-7/8) in the localization without RY *cis*-elements (*Ehd1*-none-RY). Primers in the promoters of *RAC1* (RAC1P-1/2, *RAC1*-none-RY) and *Hd1* (Hd1P-1/2, *Hd1*-none-RY) were also used as negative control. The primer sequences are available upon request.

Results

Expression of Ehd1 is suppressed by ectopically expressed OsLFL1 in W378

Mutant *W378* was first isolated from the T-DNA insertion lines constructed in our lab, with the character of late-flowering (Supplementary Fig. S1). The T-DNA flanking gene in *W378* encodes a B3 domain-containing protein. This B3 protein shows high similarity with *Arabidopsis* LEC2 and FUCSA3 (Supplementary Fig. S2) thus was named OsLFL1 (*Oryza sativa* LEC2 and FUSCA3 like 1). Besides of the B3 DNA-binding domain, a trans-activation domain was proved to be existing in the C-terminal of OsLFL1, suggesting that this protein is a B3 transcription factor. In wild-type rice Zh11, *OsLFL1* is expressed specifically in spikes and young embryos (data not shown). But in mutant *W378*, *OsLFL1* was ectopically expressed and over-expressed in both mRNA (Fig. 1) and protein levels (data not shown). When introducing the *OsLFL1*-RNAi plasmid into *W378*, the flowering of mutant was promoted by down-regulating the expression of *OsLFL1* (Supplementary Fig. S3). Over-expressing *OsLFL1* gene in rice could also reconstruct the late-flowering phenotype similar to *W378* (data not shown). All these results suggested that the overexpression of OsLFL1 was responsible for the late-flowering character of *W378*.

We carried out semi-quantitive RT-PCR to investigate expression of which genes in the flowering time pathway were influenced in mutant *W378*. Considering that many flowering time genes not only have different expression levels in short day (SD) condition and long day (LD) condition, but also vibrate during the 24 h rhythm, we sampled at different time during the course of 24 h in LD and SD conditions. In this study, 10L:14D (10 h light and 14 h dark a day) was set up as short day condition and 14L:10D (14 h light and 10 h dark a day) as long day condition. As shown in Fig. 1, besides that *OsLFL1* gene was ectopically expressed in *W378*, it had a little higher expression in SD than that in LD condition (Fig. 1). However, *Hd1*, *OsGI*, *OsLHY*, and *OsPRR1* (*OsTOC1*) [24–27] had similar expression levels in *W378* and Zh11 under both SD and LD conditions. This might suggests that OsLFL1 does not function in the Hd1 pathway. In Zh11, *Ehd1* gene [20] had a higher expression level in both SD and LD conditions, but had a similar 24 h rhythm in both conditions, with the peak at the time of transition from dark to light

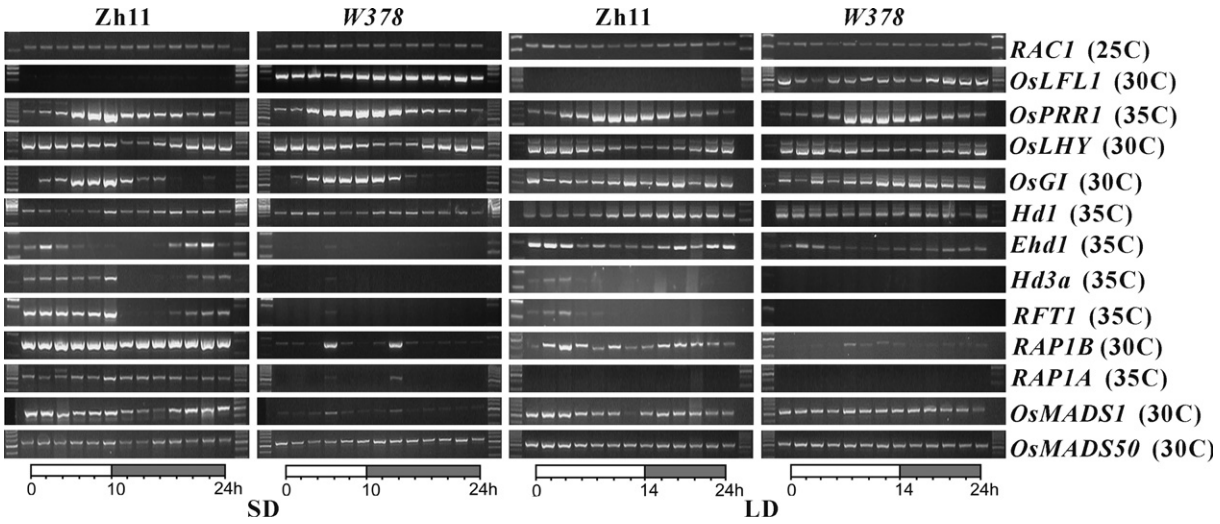

Fig. 1. Expression of rice flowering time genes in *W378* and Zh11. Leaves of mutant *W378* and Zh11 plants were collected at 20 DAS (days after sowing) on different time of a day (24 h). Gene names and cycles of RT-PCR were marked besides. GenBank Accession No. of rice flowering time genes and their RT-PCR primers were listed in Supplementary Table S1. SD, in short day condition (left); LD, in long day condition (right).

and the vale from light to dark. But in *W378*, *Ehd1* expression was obviously repressed in the 24 h rhythm, and was hardly detected in both SD and LD conditions (Fig. 1). Furthermore, in Zh11, downstream genes of *Ehd1*, including *Hd3a*, *RFT1*, *RAP1A*, *RAP1B*, and *OsMADS1*, had different expression levels during the 24 h rhythm in both SD and LD conditions. That is, their expression levels in SD condition were higher than that in LD condition, which were consistent with previous reports [20]. Under SD and LD conditions, neither *RAP1A* nor *RAP1B* had circadian rhythm. In both SD and LD conditions, the expression of *Hd3a*, *RFT1*, *RAP1A*, and *RAP1B* gene was decreased in *W378* compared with that in Zh11 (Fig. 1). *OsMADS1* gene expression was only reduced in SD in *W378* compared to Zh11. Expression of *OsMADS50* [28] was un-affected in *W378*. In summary, *Ehd1* and its putative downstream genes *Hd3a*, *RFT1*, and *OsMADSs* were repressed in *W378* under both SD and LD conditions.

Considering that *Ehd1* gene and *Hd1* gene might act in different pathway in flowering time control [20], we deduced that the *OsLFL1* gene might act in the pathways of *Ehd1* but not *Hd1*.

OsLFL1 protein interacts with RY cis-elements in the promoter of Ehd1

The B3 transcription factors are only found in plants and some of them were shown to bind the RY (RY/Sph) *cis*-elements (CATGCATG) [29,30]. Three copies of conserved RY *cis*-elements were found in the promoter region of *Ehd1* gene (http://www.dna.affrc.go.jp/PLACE/signalscan.html) (Fig. 2A). Expression analyses showed that *OsLFL1* might influence *Ehd1* and its downstream genes; considering that that OsLFL1 is putative B3 domain transcription factor, it is need to test whether OsLFL1 has the direct interaction with *Ehd1*.

Electrophoretic mobility shift assays (EMSA) was applied to determine whether OsLFL1 protein can bind to the RY *cis*-elements in *Ehd1* promoter. Three fragments containing the three respective RY *cis*-elements were isolated from the promoter of *Ehd1* and tested for interaction with OsLFL1 protein. It was found that OsLFL1 protein could bind to all of three fragments (A, B, and C) labeled with ^{32}-P (Fig. 2A). In addition, competitor assay showed that the wild-type RY (CATGCATG) containing DNA fragments (cold probes) (A, B, and C) could compete with the corresponding ^{32}P-labeled DNA fragments (hot probes). In contrast cold mutant probes (Am, Bm, and Cm), containing mutation in RY *cis*-element (CATAAATG) could not compete with the hot probes (Fig. 2A). These results indicated that the OsLFL1 protein could interact specifically with DNA fragments containing RY *cis*-elements from the *Ehd1* promoter in vitro.

In further confirmation, these wild-type and mutant DNA fragments from promoter of *Ehd1* were cloned into the yeast one hybrid reporter plasmid p178 to construct the "DNA-p178" reporter plasmid (Fig. 2B). Since the full-length OsLFL1 protein contains not only B3 DNA-binding domain but also trans-activation domain (data not shown), it was introduced into pEG202 to construct the effecter plasmid and then transformed into yeast strain EGY48 with the DNA-p178 reporter plasmid. Yeast transformants with wild DNA-p178 (A, B, and C) and OsLFL1-pEG202 plasmids turned blue in X-gal SD plates, while transformants with the mutant DNA-p178 (Am, Bm, and Cm) and OsLFL1-pEG202 did not (Fig. 2B). These results indicated that the OsLFL1 protein could interact specifically with RY *cis*-elements in the *Ehd1* promoter in yeast cells.

Immunoprecipitation-chromatin immunoprecipitation (ChIP) assay was applied to further verify the interaction between OsLFL1 protein and the *Ehd1* promoter in vivo. From expressional analysis, we know that *OsLFL1*

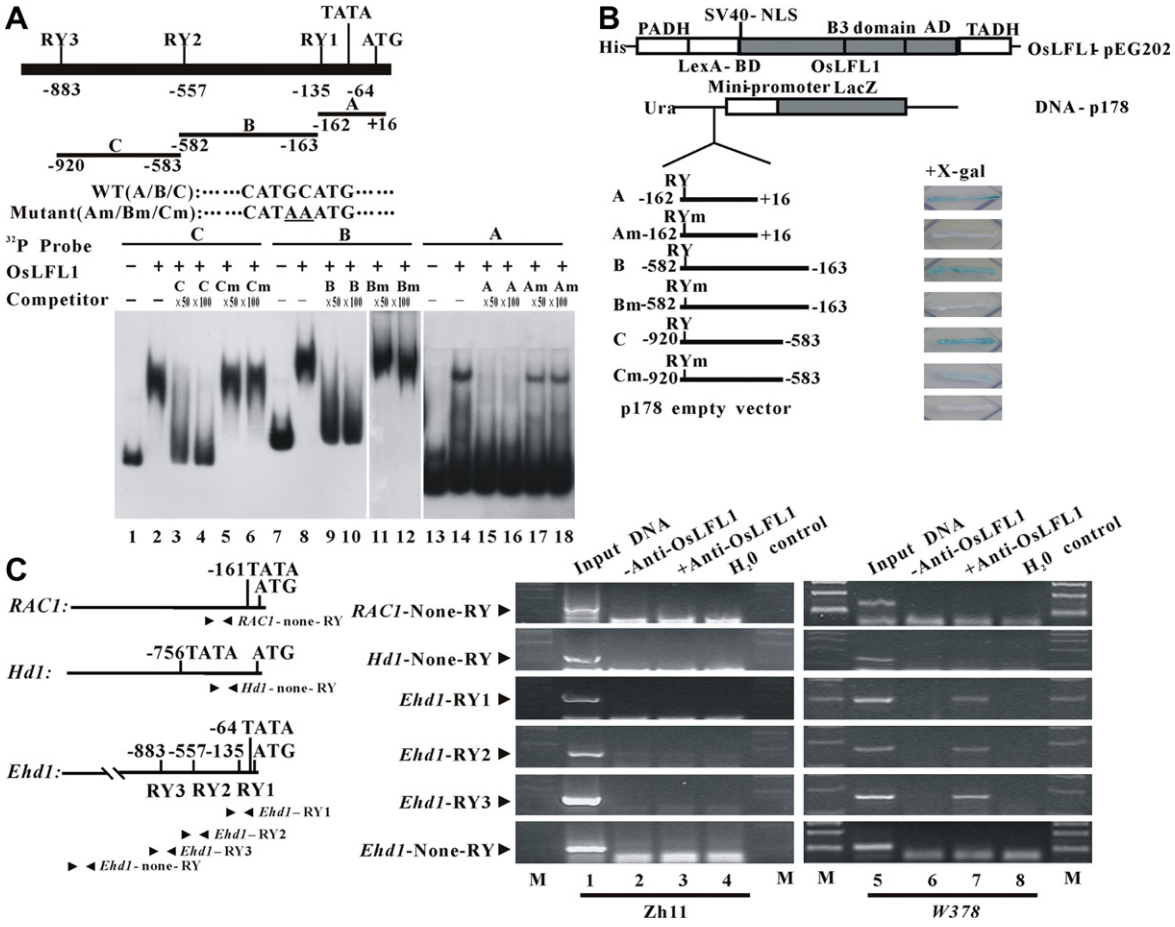

Fig. 2. Molecular interaction between OsLFL1 and the promoter of *Ehd1*. (A) Electrophoretic mobility shift assays (EMSA). Wild-type RY containing fragments: A (lanes 13 and 14), B (lanes 7 and 8), and C (lanes 1 and 2) were labeled with ^{32}P as the hot probes in binding assay. A (lanes 15 and 16), B (lanes 9 and 10), C (lanes 3 and 4) and mutant RY fragments: Am (lanes 17 and 18), Bm (lanes 11 and 12), and Cm (lanes 5 and 6) with increasing larding (50×, 100×) were used as cold probes for competing assay, respectively. Lanes 2, 3–6, 8, 9–12, 14, 15–18: adding the OsLFL1 protein, (+); lanes 1, 7, and 13: without OsLFL1 protein, (−); (B) yeast-one-hybrid assay. A, B, C and mutant Am, Bm, Cm DNA fragments were cloned into reporter plasmid p178, respectively (DNA-p178). Lanes 1–7: yeast cell transformants containing effector (OsLFL1-pEG202) and reporter (DNA-p178) were grown on X-gal containing SD medium; (C) chromatin immunoprecipitation (ChIP) assay. The fragments of the promoters of *Ehd1* (*Ehd1*-RY1, 2, 3; *Ehd1*-none-RY), *Hd1* (*Hd1*-none-RY) and *RAC1* (*RAC1*-none-RY) was amplified by PCR with templates of the ChIP products from Zh11 and *W378* (lanes 1–8); lanes 3 and 7: adding the antibody of *OsLFL1*, +anti-OsLFL1; lanes 2 and 6: without anti-OsLFL1, −anti-OsLFL1; lanes 1 and 5: adding the lysis DNA of Zh11 and *W378*, respectively, input DNA; lanes 4 and 8: negative control, H_2O control.

expressed in leaves of *W378* but not Zh11; so we took leaves samples from *W378* and Zh11 for test. Antibody of OsLFL1 protein, anti-OsLFL1, was added to pull down the OsLFL1 and DNA fragment of its target genes in both *W378* and Zh11. In *W378* sample, when anti-OsLFL1 was added, visible PCR products (*Ehd1*-RY1,2,3) were obtained with primers complementary to the RY *cis*-elements containing promoter of *Ehd1* (Fig. 2C), but in Zh11 sample, no PCR product was gotten; neither does with the primers for other regions of the *Ehd1* promoter (*Ehd1*-none-RY) and other control genes such as *RAC1* (*RAC1*-none-RY) and *Hd1* gene (*Hd1*-none-RY), which contain no RY *cis*-element in their promoter (Fig. 2C). Without anti-OsLFL1 added, none visible PCR product was obtained with any of the primers. These results suggested that the OsLFL1 protein could bind to the DNA fragment containing RY *cis*-elements in the *Ehd1* gene promoter in *W378* cells.

Discussion

The B3 DNA-binding domain (pfam02362) was identified recently and is specific for plants. *OsVP1* is the first B3 transcription factor gene cloned from rice and it is homologous to ABI3 in *Arabidopsis* [31]. In this paper, we reported the functional analysis of a novel rice B3 gene *OsLFL1*. Transgenic *Arabidopsis* plants overexpressing *OsLFL1* showed the late-flowering, dwarf and small leaves and leaf-like petals phenotypes (data not shown), which were similar to those ABI3 and FUSCA3 over-expression transgenic plants [32,33], suggesting that the *OsLFL1* gene might have similar function to those B3 domain transcription factor genes in *Arabidopsis*.

In our pervious work, it was found that the overexpression of *OsLFL1* was responsible for the late-flowering character of mutant *W378* (Supplementary Fig. S3). In this study, several flowering time genes such as *Ehd1*, *Hd3a*,

RFT1, and *OsMADS* genes were found to be remarkably reduced in *W378* mutant in the 24 h rhythm under SD and LD conditions (Fig. 1). In rice, since *Ehd1* is a flowering promoting gene in rice, acting upstream of *Hd3a*, *RFT1*, and *OsMADS* genes, down-regulation of *Ehd1* in *W378* might be result in the late-flowering character. Up to now, no upstream genes or regulatory factors of *Ehd1* were reported in rice. In this study, expression analysis suggested that over-expression of *OsLFL1* might confer to the down-regulation of *Ehd1*. Furthermore, *Ehd1* could be up-regulated in *W378* by down-regulating *OsLFL1* when introducing *OsLFL1*-RNAi to this mutant (data not shown). These results suggested that OsLFL1 might be one of transcriptional repressor upstream of *Ehd1*.

B3 proteins including ABI3/VP1, LEC2, and FUSCA3 could bind specifically to the RY *cis*-elements and regulate expression pattern of their respective target genes [29,34,35]. The RY *cis*-element was the 8 bps motif (CATGCATG) conserved in plants. In rice genomic, the probability of RY *cis*-element was very low, especially in the promoter region (data not shown) and so that we deem that they play important roles in rice (Fig. 2A). In this study, EMSA assay showed that OsLFL1 could bind specifically to DNA fragments containing RY but not to those containing mutant RY (Fig. 2A). Yeast-one-hybrid analysis verified further the interaction between OsLFL1 and RY motifs in yeast cells (Fig. 2B). In addition, ChIP assay also indicates that OsLFL1 could bind to the promoter region of *Ehd1* in mutant *W378* (in vivo). Those results suggest strongly that OsLFL1 can bind to the promoter of *Ehd1*, specifically to the RY motifs (Fig. 2C). Therefore, we deduced that over-expression of OsLFL1 suppressed *Ehd1* expression by direct binding to the RY motifs in the promoter, which caused the late-flowering phenotype in *W378*.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2007.06.041.

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