Journal of Plant Physiology 165 (2008) 876–885

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JOURNAL OF PLANT PHYSIOLOGY

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Overexpression of transcription factor *OsLFL1* delays flowering time in *Oryza sativa*☆

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Received 29 May 2007; received in revised form 11 July 2007; accepted 12 July 2007

KEYWORDS
Late flowering;
B3 DNA-binding domain;
Overexpression;
OsLFL1;
Oryza sativa

Summary

Flowering time is regulated by genetic programs and environment signals in plants. Genetic analysis of flowering time mutants is instrumental in dissecting the regulatory pathways of flower induction. Genotype *W378* is a rice (*Oryza sativa*) late-flowering mutant selected from our collections of T-DNA insertion line. The T-DNA flanking gene in mutant *W378* codes OsLFL1 (*O. sativa* LEC2 and FUSCA3 Like 1), a putative B3 DNA-binding domain-containing transcription factor. In wild-type rice *OsLFL1* is expressed exclusively in spikes and young embryos, while in mutant *W378* it is ectopically expressed. Introduction of *OsLFL1*-RNAi into mutant *W378* successfully down-regulated *OsLFL1* expression and restored flowering to almost normal time, indicating that overexpression of *OsLFL1* confers late flowering for mutant *W378*. The flowering-promoting gene *Ehd1* and its downstream genes are all down-regulated in *W378*. Thus, overexpression of *OsLFL1* might delay the flowering of *W378* by repressing the expression of *Ehd1*.
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Introduction

It is essential for plants to transit from vegetative growth to reproductive growth for successful sexual reproduction. More than 50 flowering time genes were identified with forward and reverse genetic approaches (Putterill et al., 2004). The flowering time in rice is also called heading date (Hd). Approximately 15 quantitative trait loci controlling

Abbreviations: T-DNA, transferred DNA; RT-PCR, reverse transcriptase PCR; RNAi, RNA interference; GUS, β-glucuronidase; GFP, green fluorescent protein; X-gal, 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside

☆The nucleotide sequence for *OsLFL1* has been deposited in GenBank under accession no. EF521182.

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0176-1617/$ - see front matter © 2007 Elsevier GmbH. All rights reserved.
doi:10.1016/j.jplph.2007.07.010

Hd have been identified in rice (Yano et al., 2001; Doi et al., 2004). Up to now, 4 genes of them (*Hd1*, *Hd3a*, *Hd6* and *Ehd1*) have been characterized. *Hd1* was orthologous to *CONSTANS* (*CO*) in *Arabidopsis thaliana*, but with different function (Yano et al., 2000). Hd1 promotes rice flowering time under short-day conditions but represses flowering under long-day conditions (Yano et al., 2000; Izawa et al., 2002). *Hd3a* is orthologous to *Arabidopsis FLOWERING LOCUS T* (*FT*) and directly regulated by Hd1 (Kojima et al., 2002; Izawa et al., 2002). *Hd6* encodes the α subunit of a protein kinase CK2, which might act upstream of *Hd1* (Takahashi et al., 2001). *Ehd1* gene codes a novel B-type response regulator and presents as a rice flowering-promoting gene under short-day conditions (Doi et al., 2004). Ehd1 and Hd1 both act upstream of rice *FT*-like genes (*Hd3a*, *RFT1*, etc.) and *OsMADS* genes. However, mutant and transgenic analyses suggest that Ehd1 and Hd1 might act in two different pathways for photoperiod control of rice flowering (Doi et al., 2004). Other flowering genes in photoperiod pathway, such as *OsGI* (*GIGANTEA*), *OsPRR1* (*TOC1*) and *OsLHY* (*LHY*), were cloned with sequence similarity to the respective *Arabidopsis* genes, and might function upstream of *Hd1* (Hayama et al., 2001; Izawa et al., 2002). But the upstream genes or regulating genes of *Ehd1* gene have not been reported to date.

Several rice MADS genes were also proved to be involved in controlling flowering time. *OsMADS14* (*RAP1B*) and *OsMADS15* (*RAP1A*) are rice orthologs for the *Arabidopsis* class A gene *APETALA1* (*AP1*) and class C gene *AGAMOUS* (*AG*), and play important roles in floral transition (Kyozuka et al., 2000). *OsMADS1*, also homologous to *AP1*/*AG*, functions in rice floret organ development (Prasad et al., 2005). Ectopic expression of *OsMADS1* promotes flowering time remarkably (Chung et al., 1994). Overexpression of another *AP1*/*AG* homolog rice gene, *OsMADS18*, also induced early flowering (Fornara et al., 2004). *OsMADS50*, the homolog of *Arabidopsis SOC1*/*AGL20*, might act as an important flowering activator upstream of *OsMADS1*, *OsMADS14*, *OsMADS15*, *OsMADS18* and *Hd3a* (Lee et al., 2004). In addition, *SE5*, encoding a putative heme oxygenase, was isolated from rice early-flowering mutant *photoperiodic sensitivity 5* (*se5*) and might function in phytochrome chromophore biosynthesis, suggesting that phytochromes confer photoperiodic control of flowering in rice (Izawa et al., 2000). *OsPIPK1*, encoding a 792-aa putative phosphatidylinositol 4-phosphate 5-kinase, was also identified to be involved in controlling rice heading through regulation of *Hd1* and *RFT1* (Ma et al., 2004).

A late-flowering rice mutant *W378* was isolated from the transferred DNA (T-DNA) insertion lines in our lab. It was proven that the late-flowering phenotype was co-segregated with T-DNA insertion (Wang et al., 2000; Chen et al., 2004). In this study, we reported that the T-DNA was inserted in *OsLFL1* gene (*Oryza sativa LEC2 and FUSCA3 Like 1*) in *W378*. *OsLFL1* encodes a putative B3 domain-containing transcription factor. Transgenic results suggested that overexpression of *OsLFL1* conferred the late flowering of *W378*. In addition, the expressional down-regulation of flowering-promoting gene *Ehd1* in mutant *W378* suggested that *OsLFL1* might act upstream of *Ehd1*.

B3 domain (pfam02362) as in *OsLFL1* was identified recently and is specific for plants. It was first reported in proteins abscisic acid-insensitive 3 (ABI3) from *Arabidopsis* and viviparous 1 (VP1) from *Zea Mays*, and was identified as DNA-binding motif (McCarty et al., 1991; Giraudat et al., 1992; Suzuki et al., 1997). LEC2 and FUSCA3 are both B3 domain transcription factors involved in embryo development and seed maturation (Parcy et al., 1997; Stone et al., 2001; Tsuchiya et al., 2004).

Materials and methods

Plant species and growth conditions

Rice Zh11 (*Oryza sativa L. subsp. japonica cv.* Zhonghua No. 11) was used as wild type. *Agrobacterium*-mediated transformation was performed as described previously (Lee et al., 1999). Mutant *W378* was selected from rice Zh11 T-DNA insertion mutant line collections. Zh11, mutant *W378* and transgenic plants were grown in green houses (with 10, 12 or 14 h light a day, ~383 μmol m^{-2} s^{-1}) for phenotype analyses.

RNA analysis

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

Protein analysis

Proteins were extracted from Zh11 and *W378* leaves, separated on 8% SDS-PAGE and transferred to PVDF membranes (Amersham, GE healthcare). Protein gel blots were performed as described in the protocol of Amersham ECL Plus Western Bloting Detection reagents (Amersham, GE healthcare), using a 1:5000 dilution of the anti-OsLFL1 rabbit antiserum and a 1:10,000 dilution of a horseradish peroxidase-linked species-specific whole antibody of the

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