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Chapter 2

Functions of OsDof24 in determining flowering time

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Abstract

OsDof24 belongs to the so-called Dof (DNA-binding with one finger) transcription factor family which is involved in a variety of biological processes in plants. Transgenic rice overexpressing *OsDof24* showed a delay of flowering time under photoperiod of 12h light/12h. RT-PCR and qPCR profiling on these plants showed that the flowering time genes *OsMADS1*, *OsMADS14*, *Hd3a* and *RFT1* were down-regulated, whereas *OsMADS50* was up-regulated, and the expression level of *Hd1*, *OsMADS15*, *OsMADS18*, *OsMADS51* and *Ehd1* were not changed. Bioinformatics analyses of the *OsMADS1*, *OsMADS14*, *Hd3a*, *RFT1* and *OsMADS50* promoters confirmed the presence of Dof binding sites. Based on these results, we hypothesize that *OsDof24* may be involved in determining flowering time by down-regulating the expression of several flowering time genes, especially *Hd3a* and *RFT1* which are two key genes in the flowering time pathway in rice.

Introduction

One of the most important traits in rice cultivation is flowering time because it determines if the crop fits in the local season. The exact moment of flowering time is determined by the genetic background and the interactions with environmental factors (Searle and Coupland 2004; Baurle and Dean 2006; Imaizumi and Kay 2006; Yu *et al.* 2011). One of the most important environmental factors that affect flowering time, apart from temperature, is the change in day length (photoperiod) (Imaizumi and Kay 2006). According to photoperiodic responses, plants are grouped into three classes: long-day (LD) plants, in which flowering occurs when the day becomes longer than a crucial length; short-day (SD) plants, in which flowering occurs when the day becomes shorter; and day-neutral plants in which flowering is not regulated at all by day length (Salisbury 1985). Normally if the light is more than 14 hours during a day, it is considered a long day (LD) condition, and if the dark is more than 14 hours, it is considered a short day (SD) condition. There are different factors influencing the photoperiodic pathway such as photoreceptors, the circadian clock and floral integrator genes (Simpson and Dean 2002; Hayama *et al.* 2003; Imaizumi and Kay 2006). In Arabidopsis, the *GI-CO-FT* related pathway is a critical mechanism in the photoperiod pathway (Putterill *et al.* 1995; Valverde *et al.* 2004). *GIGANTEA* (*GI*) integrates cellular signals from light sensory transduction and circadian clock and then activates *CONSTANS* (*CO*). Under LD conditions, *CO* promotes flowering by regulating transcription of *FLOWERING LOCUS T* (*FT*) which encodes a mobile protein called florigen which promotes flowering under both SD and LD conditions (Samach *et al.* 2000; Tiwari *et al.* 2010; Kardailsky *et al.* 1999). *CYCLING DOF FACTOR 1* (*CDF1*), a Dof transcription factor, is expressed in the same tissues with *CO* and it binds to the *CO* promoter thereby acting as repressor of *CO* transcription. In turn FLAVIN-BINDING, KELCH REPEAT,F-BOX1(FKF1) controls the expression of *CO* by mediating the degradation of CDF1 (Imaizumi *et al.* 2005).

The GI-CO-FT regulatory module controlling flowering time is conserved as OsGI-Hd1-Hd3a (Yano *et al.* 2000; Hayama *et al.* 2002; Kojima *et al.* 2002) in rice. *OsGI*, the ortholog of Arabidopsis *GI* regulates expression of *Hd1* (*Heading date 1*) and *OsMADS51* (Hayama *et al.* 2002, 2003; Kim *et al.* 2007). Under SD conditions, Hd1 promotes flowering by up-regulating expression of *Heading date 3a* (*Hd3a*), but under LD conditions it suppresses the expression of *Hd3a* thereby delaying flowering time (Kojima *et al.* 2002; Hayama *et al.* 2003). *Hd3a* in fact encodes for florigen, the long searched for mobile flowering signal, and promotes floral transition by regulating the expression of its downstream genes *OsMADS14* and *OsMADS15* (Kojima *et al.* 2002; Tamaki *et al.* 2007). *RICE FLOWERING LOCUS T1 (RFT1*), a gene homologous to *Hd3a*, also encodes a mobile flowering signal and promotes floral transition under SD condition. The expression of *RFT1* is normally very low plants but increased in *Hd3a* RNAi plants. Both *Hd3a* and *RFT1* are essential for flowering in rice (Komiya *et al.* 2008). *Early heading date 1* (*Ehd1*) encoding a B-type regulator promotes rice flowering under SD condition (Doi *et al.* 2004). There is no orthologous gene in the *Arabidopsis* genome of *Ehd1*, so there are differences in the pathway controlling flowering time between these species. In this pathway, *Ehd2*, a putative transcription factor with zinc finger motifs, acts as a flowering promoter by up-regulating *Ehd1* under SD condition (Matsubara *et al.* 2008). *OsMADS51* is a short day flowering promoter functioning upstream of *Ehd1*, *OsMADS14* and *Hd3a* (Kim *et al.* 2007). *OsMADS50* acts as an important flowering activator under LD condition of the heading date related genes *OsMADS1*, *OsMADS14*, *OsMADS15*, *OsMADS18* and *Hd3a* (Lee *et al.* 2004; Komiya *et al.* 2009). Recently, it was reported that *OsDof12* regulates flowering time by regulating expression of *Hd3a* and *OsMADS14* (Li *et al.* 2009). In tomato, Dof transcription factors (SlCDFs) were implicated in abiotic stress tolerance and flowering time (Corrales *et al.* 2014). Taken together, it is clear that flowering time is a complex process determined by many different genes with activating or repressing functions.

Dof proteins are members of a plant-specific transcription factor family (Yanagisawa 2004), which share a highly conserved DNA-binding domain (Yanagisawa 2000). The name of Dof is coined from DNA-binding with one C2-C2 zinc finger (Umemura *et al.* 2004). There are two major domains in the Dof transcription factors: an N-terminal conserved DNA-binding domain and a C-terminal domain for transcriptional regulation (Kang and Singh 2000; Yanagisawa 2000). The Dof domain is known to be a bi-functional domain which participates in not only DNA-binding but also in protein-protein interactions (Yanagisawa 1997). The highly conserved Dof domains in different Dof proteins suggested that Dof proteins display similar DNA-binding specificity. In fact, an AAAG motif or its reversibly orientated sequence, CTTT, are always the targets for Dof proteins, except for a pumpkin Dof protein (AOBP) which recognizes an AGTA repeat as its binding motif (Yanagisawa 2000). The highly variable amino acid sequences outside the Dof domain suggested that the functions of Dof proteins might be diverse. Since the first Dof protein, maize

Dof1, was identified in maize (Yanagisawa 2000), numerous Dof proteins have been reported playing important roles in diverse plant-specific processes such as response to plant hormones including gibberellin (Mena *et al.* 2002), auxin (Kisu *et al.* 1997, 1998; Baumann *et al.* 1999) and salicylic acid (Kang and Singh 2000; Kang *et al.* 2003); plant development including seed germination (Dong *et al.* 2007; Moreno-Risueno *et al.* 2007); stomata guard cell opening (Plesch *et al.* 2001); light responses (Yanagisawa 2000) and grain size control (Masao *et al*. 2009).

In the rice genome 30 putative Dof transcription factors have been identified (Lijavetzky *et al.* 2003), but so far only *OsDof3* and *OsDof12* were studied in detail (Yano *et al.* 2000; Li *et al.* 2009). In this study, we identified *OsDof24*, a Dof transcription factor, and investigated its functions in determining the heading date. *OsDof24* was assigned to the group C-type Dof transcription factors, which are related to seed maturation, seed germination and vascular development (Shigyo *et al.* 2007). In this chapter, we demonstrate that *OsDof24* is a key regulator of flowering time by down-regulating the flowering time genes *OsMADS1*, *OsMADS14*, *Hd3a* and *RFT1*.

Results

OsDof24 belongs to the rice Dof transcription factor family

OsDof24 is one of the 30 genes encoding the Dof transcription factor family in rice (Lijavetzky *et al.* 2003; Riano-Pachon *et al.* 2007). The gene encoding OsDof24 obtained from hybrid rice combination Liangyou 2186 is 831 bp long which is the same as a TIGR prediction (LOC_Os08g38220 in MSU osa1 release 7) and encodes for a 276 amino acid protein. There is no intron in the *OsDof24* genomic sequence. Figure S1 shows the sequence and structure of OsDof24 including the Dof domain and the four cysteine residues characteristic for the Dof zinc finger.

To confirm that OsDof24 is a nuclear-localized transcription factor, a GFP-tagged fusion protein under control of the CaMV 35S promoter was made, with the GFP ORF fused at the C-terminus of the full-length OsDof24 protein (construct 35S::GFP-OsDof24). This construct was introduced into rice protoplasts by PEG-mediated transient transformation and analyzed using CSLM, where a 35S::GFP construct was used as control. As shown in Figure 1A, a GFP signal was detected specifically in the nucleus of 35S::OsDof24-GFP transformed cells, whereas in the control, the GFP signal was located primarily in the cytoplasm. These data support that OsDof24 is a nuclear-localized protein.

Expression profile of *OsDof24*

The expression profile of *OsDof24* was analyzed using qPCR in wild-type rice and by GUS staining in transgenic plants equipped with a promoter-GUS reporter construct. The expression level of *OsDof24* was found highest in two week old seedlings and lowest in stems of mature plants, respectively (Figure 1B) Expression at different levels was seen in penultimate leaves, leaf sheath, stems, roots and panicles.

Using the *OsDof24* promoter-*GUS* construct, GUS activity was detected in embryogenic calli, flag leaves, panicles, shoot apical meristem (SAM). GUS activity was also visible in developing seeds and peaked at 10 DAF (days after fertilization). There are also GUS signals in stem, roots and two days old germinating seeds. Cross-sections of leaf tissue and the SAM showed that the GUS signal was highest in cells associated with the vascular bundles (Figure 1C). In panicles, *OsDof24* was expressed in the palea and lemma, which enclose the other flower organs and the developing grain (Figure 1C). The GUS activity in ProOsDof24::GUS transgenic rice is in accordance with the expression profile of *OsDof24* as analyzed using qPCR.

Delay of flowering time in *OsDof24* overexpressing transgenic rice

To further elucidate the function of *OsDof24*, transgenic overexpression plants were made. For this, a binary vector, *ProGOS2::OsDof24*, was constructed by expressing the *OsDof24* under control of the constitutive *GOS2* promoter (De Pater *et al.* 1992; Ouwerkerk *et al.* 2001). Before phenotyping, single copy plants originating from three independent callus lines were genotyped using qPCR. As shown in Figure 2c, the qPCR analysis of the T_1 plants showed that upregulation of expression of *OsDof24* ranged from 3 to 10 times. Flowering time was recorded as the first day the first flower appeared compared to the day the plants were seeded. Plants from all three lines were phenotyped in the greenhouse, and showed two weeks later flowering than the controls, which were non-transgenic azygous plants segregated from the T_0 generation (Figure 2a). Statistical analysis showed that the *OsDof24-OX* plants were flowering at least two weeks later compared to the controls. For instance, line OX1 was flowering 38 days later than the control and lines OX2 and OX3 were flowering respectively 15 and 20 days later. Furthermore, another obvious observation was that the leaves of the *OsDof24-OX* plants were growing only in one plane whereas normally wild type rice leaves grow in all directions (Figure 2b). Obviously, *OsDof24-OX* T_1 plants were smaller than the controls, (Figure 2a). The average height of the *OsDof24-OX* plants was 14 cm to 44 cm shorter (26 cm in average) than the control. The internode length was 8 cm to 17 cm shorter but the leaf number per tiller was at least three more than the control (Figure 2, Table 1). Thus although the plants had more leaves and thus (inter)nodes, they were still shorter because internode length was reduced. These results indicated that *OsDof24* does not only have a role in determining flowering time but also in other major traits such as plant height, leaf number and internode length.

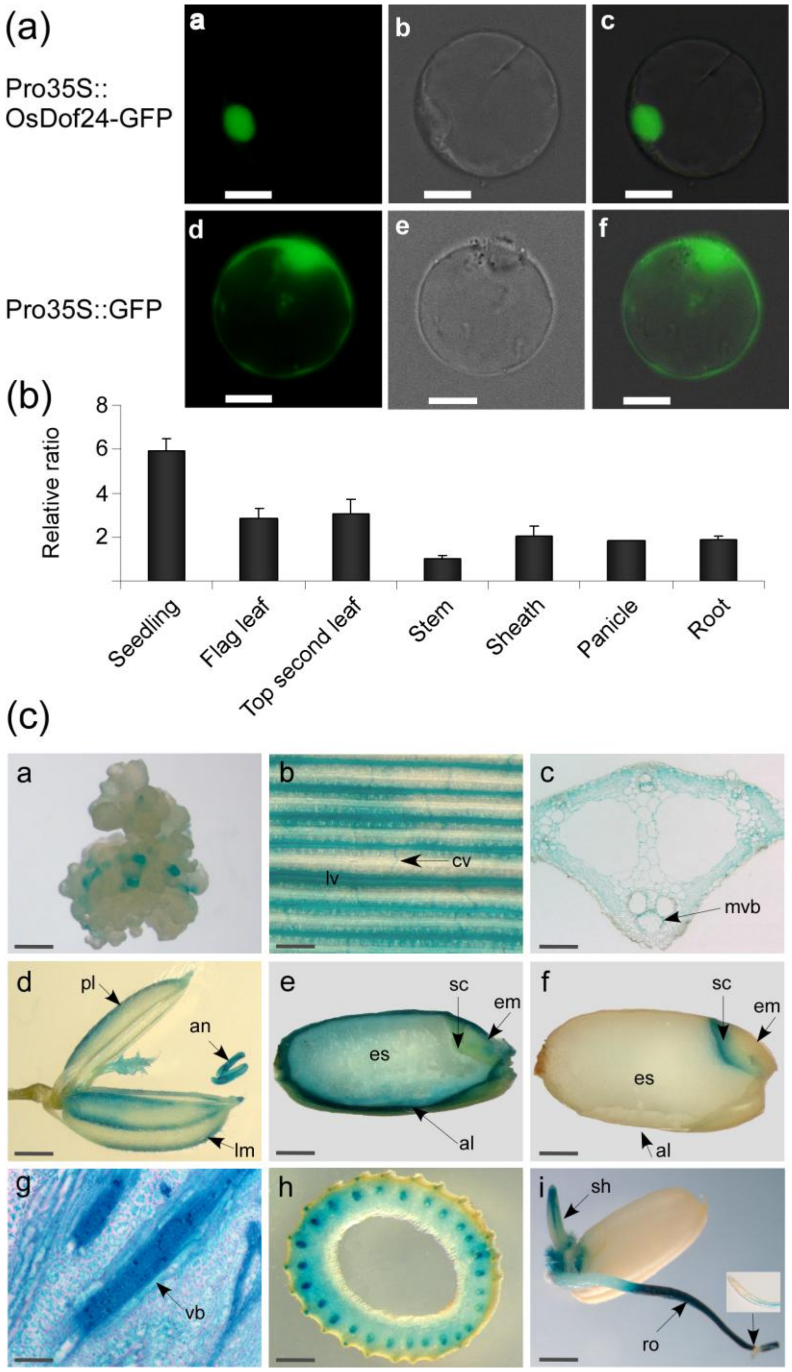

Figure 1. Subcellular localization of a GFP-tagged OsDof24 protein in rice protoplasts, and expression profile of *OsDof24*.

(a) Protoplasts were transiently transformed with either construct Pro35S::OsDof24-GFP (a, b and c) or Pro35S::GFP (d, e and f). After culturing overnight, protoplasts were observed with fluorescence (a, d) and bright field microscopy (b, e). A merged panel of a and b is shown in panel (c), and the combination of d and e is shown in panel (f). Scale bars represent 2 μm.

(b) qPCR analysis of *OsDof24* expression in seven different tissues. Two week old seedlings, 10 DAF (day after flowering) flag leaves, penultimate leaves, stems, sheath, panicles and roots were analyzed for *OsDof24* expression using qPCR. *Ubi* was used as a control for normalization of cDNA quantity. Bars represent means and standard deviation (n=3 independent qPCRs).

(c) Histochemical localization of *OsDof24* promoter-*GUS* expression in transgenic rice. Embryogenic callus (a), flag leaves (b), cross section of flag leaves (c), immature spikelet (d), seeds at 10 DAF (e), 15 day after flowering seeds (f), longitudinal section of the SAM (g), vascular bundles (g), cross section of stems (h) and after two days of germination (i). al, aleurone layer; an, anther; cv, commissural vein; em, embryo; es, endosperm; lm, lemma; lv, longitudinal vein; mvb, main vascular bundle; pl, palea; sh, shoots; ro, roots; vb, vascular bundle. Scale bars in a, b, d, e, f and i represent 10 mm, in c and g 100 µm and in h 10 µm, respectively.

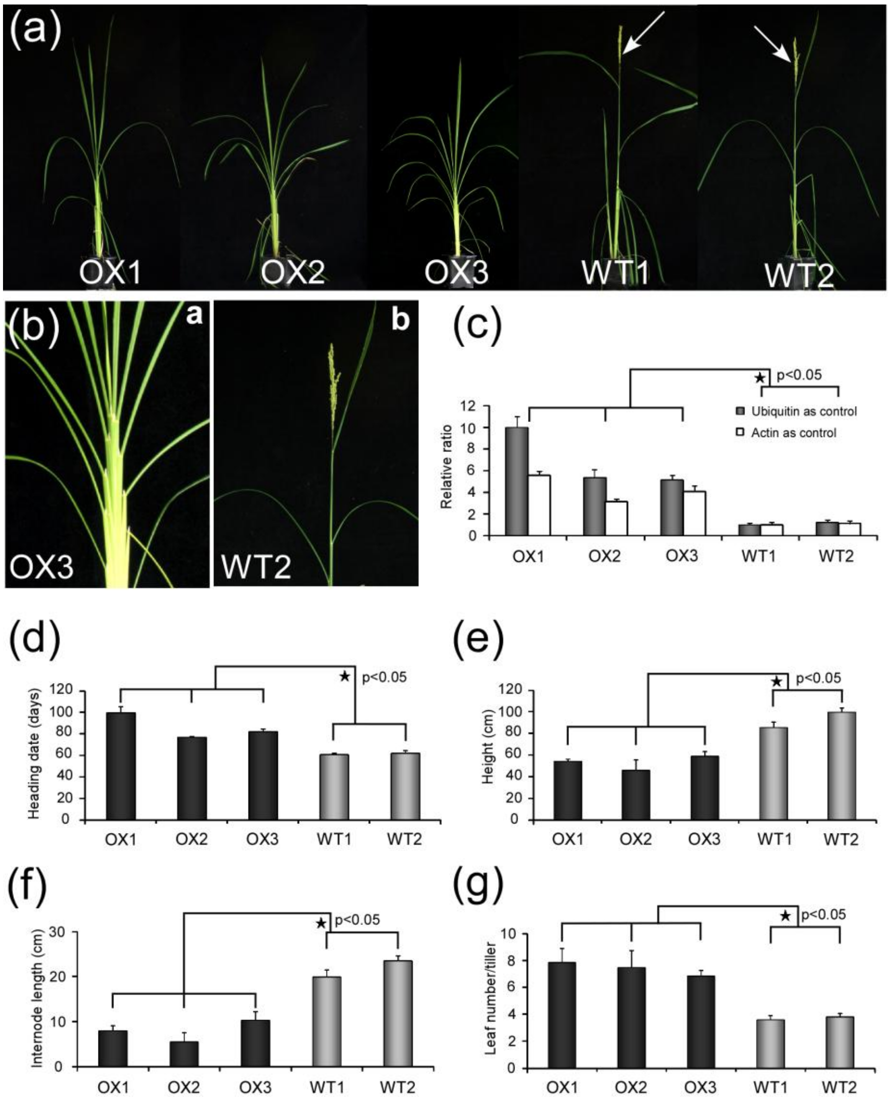

Figure 2. Phenotypical characterization and expression levels of *OsDof24* in overexpression plants and control lines.

(a) Late flowering phenotype of *OsDof24* overexpressing transgenic lines in comparison to control plants. Panicles are indicated by arrows.

(b) Two dimensional leaf distribution and late flowering phenotype of the OsDof24-OX lines. Magnified picture of OsDof24-OX line 3 is shown in panel (left), and a control plant is shown in panel (right).

(c) qPCR results on OsDof24-OX lines 1 to 3. *Ubiquitin* and *Actin* gene expression levels were used as internal controls for normalization of cDNA quantities. Bars represent means and standard deviation (n=3 independent qPCRs).
(d) Flowering time of OsDof24-OX plants and control lines. Flowering time was determined as the number of days from germination (DAG) to the first day that a panicle started flowering.
(e) Height of OsDof24-OX plants and control lines. Height of plants was measured from the soil till the top of the panicle at 120 DAG.
(f) Internode length of OsDof24-OX plants and control lines at 120 DAG.
(g) Average leaf number per tiller of OsDof24-OX plants and control lines at 120 DAG. For each experiment, three or more plants were included. Bars represent means and standard deviation (n ≥3 independent plants measured for each independent transgenic or control line). The data were analyzed using ANOVA followed by Bonferroni corrections. Asterisks indicate significant differences ($p<0.05$) compared with the untransformed controls.

Plants	Heading date (day)	Height (cm)	Leaf number/tiller	Internode length (cm)
OX1	100.00 ± 5.24	54.57 ± 2.05	7.86 ± 1.02	8.00 ± 1.15
OX2	77.00 ± 0.82	46.00 ± 9.80	7.50 ± 1.22	5.50 ± 2.04
OX3	82.57 ± 2.54	59.00 ± 4.37	6.86 ± 0.40	10.43 ± 1.79
WT1	60.80 ± 1.49	85.80 ± 4.82	3.60 ± 0.32	20.00 ± 1.47
WT2	62.00 ± 2.48	100.00 ± 4.08	3.80 ± 0.26	23.60 ± 1.05

P＜0.05

Table 1. Overview of phenotyping results from *OsDof24-OX* and wild type plants. Flowering time was calculated from the germination to the day that the first floret opened. Plant height was measured from the soil to the top of the highest panicle at 120 DAG. Internode length and leaf number per tiller were also counted at 120 DAG. From every line, three or more plants were measured. The data were analyzed using ANOVA followed by Bonferroni. Asterisks indicate significant differences ($p<0.05$) compared with the untransformed controls.

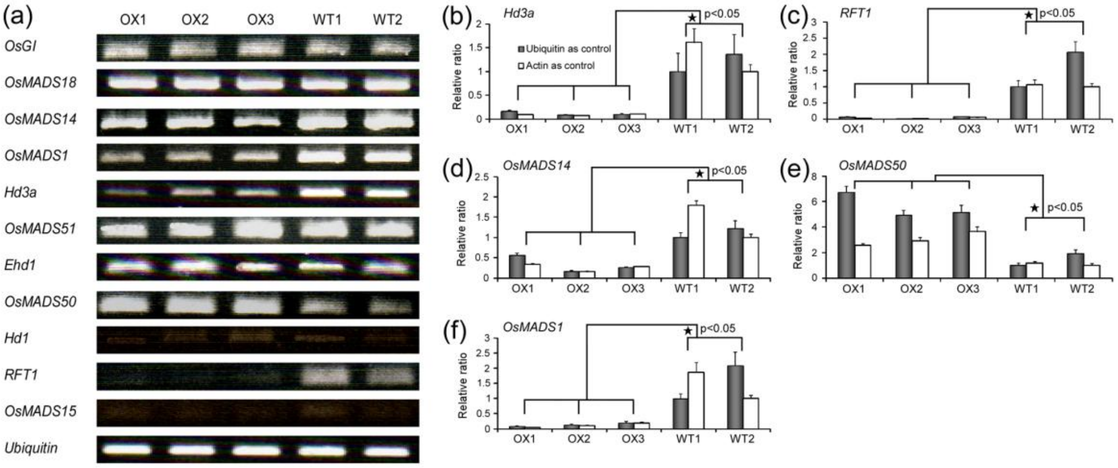

Figure 3. Expression profile of flowering time genes in control and OsDof24-OX plants and qPCR expression profiling of the flowering time genes.

(a) Expression level of *OsGI*, *OsMADS18*, *OsMADS14*, *OsMADS1*, *Hd3a*, *OsMADS51*, *Edh1*, *OsMADS50*, *Hd1*, *RFT1* and *OsMADS15* were analyzed by semi-quantitative RT-PCR. *Ubi* gene expression was used as control for normalization of cDNA quantity.
(b), *RFT1* (c), *OsMADS14* (d), *OsMADS50* (e) and *OsMADS1* (f) in control and OsDof24-OX plants..Both *Ubiquitin* and *Actin* expression levels were used as controls for normalizing cDNA quantities. Vertical bars indicate the standard deviation of three technical replicates for each sample.

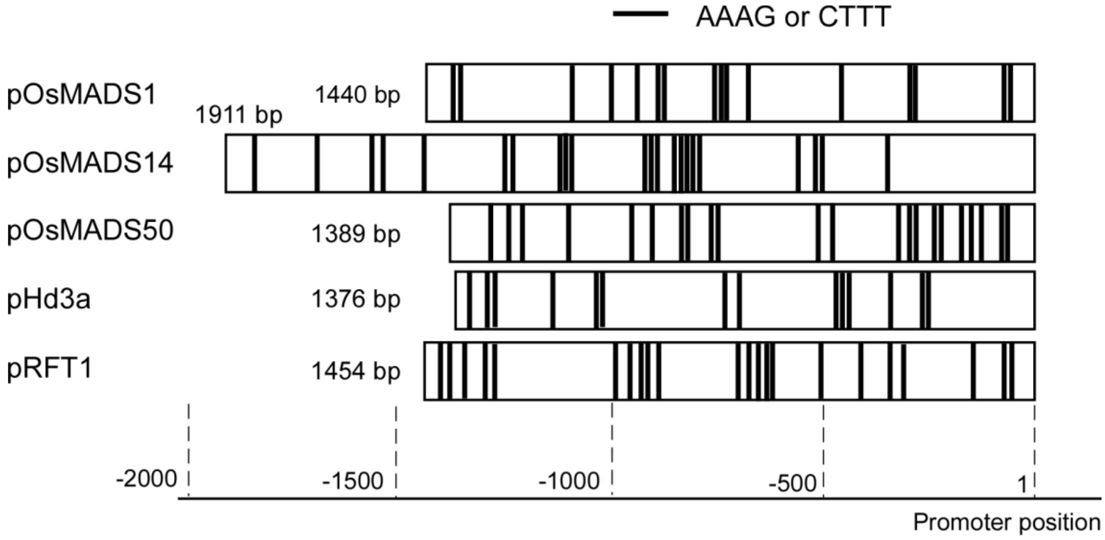

Figure 4. The distribution of the Dof binding motif in the promoters of flowering time-related genes *OsMADS1*, *OsMADS14*, *OsMADS50*, *Hd3a* and *RFT1*.

Downregulation of OsDof24 does not result in phenotypical changes

Given the results obtained with the overexpression plants, we were also interested to see the effect of a loss-of-function approach either using mutants or RNAi lines. We checked available mutant collections in RiceGE (http://signal.salk.edu/cgi-bin/RiceGE) and identified one T-DNA mutant line in *OsDof24* (PEG_3A00724 from the Postech collection (Jeong *et al.* 2006)). BLAST analysis showed that the T-DNA was inserted 640 bp upstream of the ATG. Using Southern blotting, homozygous and heterozygous plants of this mutant were identified. However, homozygous mutant plants did not show any obvious phenotype and we also confirmed that expression was not down-regulated (results not shown). Thus apparently, this mutation did not have any effect on expression. Subsequently, a binary vector construct enabling an RNAi approach was made and transgenic plants were generated. The expression of *OsDof24* was checked and was found down-regulated in two of the transgenic lines although it was still expressed to some level (Figure S2). However, the RNAi plants did not show any obvious phenotypical difference (Figure S3), neither in architecture nor in flowering time.

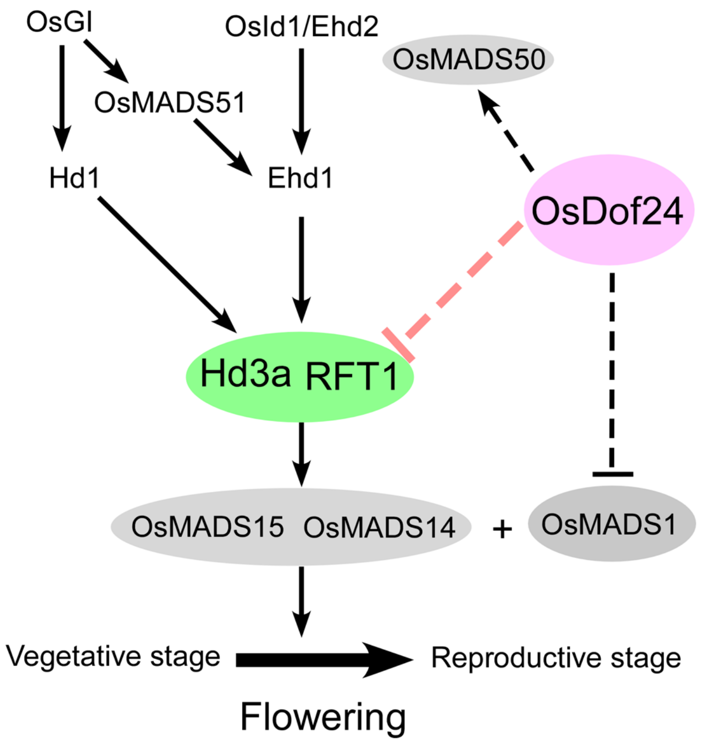

Figure 5. Model of the flowering time pathway under a 12 h light/12 h dark condition in rice. *OsDof24* overexpression down-regulates expression of *OsMADS14*, *OsMADS1*, *Hd3a* and *RFT1*, but does not affect *Hd1* and *Ehd1*. According to this model, *Hd3a* and *RFT1* function as activators which are critical for flowering time and they also regulate expression of *OsMADS14*. *OsDof24* act as a repressor of flowering time by downregulating expression of *Hd3a* and *RFT1*.

Effects of *OsDof24* overexpression on flowering time genes

Genes that have been reported to determine flowering time in rice include *Ehd1, Hd3a, Hd1, RFT1, OsMADS50, OsMADS14, OsMADS15, OsMADS18, OsMASD51, OsMADS1* and *OsGI* and this group will likely increase (Kojima *et al.* 2002; Doi *et al.* 2004; Lee *et al.* 2004; Kim *et al.* 2007; Komiya *et al.* 2008;). To study the link between *OsDof24* and these regulators, we analyzed their expression levels using RT-PCR in flag leaves at 10 DAF in control and *OsDof24-OX* plants. As shown in Figure 3, the expression of *Ehd1*, *Hd1*, *OsMADS18*, *OsMADS15*, *OsMADS51* and *OsGI* were not significantly affected, but the transcription level of *OsMADS50* was upregulated in the *OsDof24-OX* plants, whereas the expression level of *Hd3a*, *OsMADS1*, *OsMADS14* and *RFT1* was down-regulated.

For a subset of flowering time genes, the expression level was further analyzed using qPCR (Figure 3). The results showed that *Hd3a*, *RFT1*, *OsMADS14* and *OsMADS1* were down-regulated in the *OsDof24*-OX plants compared to the control plants, whereas *OsMADS50* was upregulated thereby confirming the RT-PCR results. All these data suggested that *OsDof24* might influence rice flowering time by regulating the expression of flowering time genes.

Discussion

Dof proteins are plant-specific transcription factors, with known functions as transcriptional activator or repressors playing critical roles during plant growth and development (for a review see Yanagisawa 2004). Among the 30 Dof transcription factors in the rice genome (Lijavetzky *et al.* 2003), so far only *OsDof3* (Washio 2001, 2003) and *OsDof12* (Li *et al.* 2009) have been studied in more detail. In this study, we report on the functional characterization of *OsDof24* and propose a role in regulating flowering time, plant height and leaf growth.

Expression profiling showed that *OsDof24* was expressed in a variety of tissues including two weeks seedlings, 10 DAF leaves, stems, sheaths, panicles and in roots. Furthermore, GUS stainings of transgenic rice containing construct *ProOsDof24::GUS* confirmed expression in seedling and also in leaves, stems, panicles and roots of the plants in filling stage, which is in accordance with the expression profile as determined by qPCR. In addition, we also observed a strong GUS signal in germinating seeds of two days old but we do not find the GUS signal in immature seeds. Like most transcription factors, OsDof24 is targeted to the nucleus in rice. The expression profile of *OsDof24* indicates that it is important in different stages of different tissues during rice growth and development.

To describe the functions of *OsDof24* in more detail, we generated *OsDof24* overexpressing and RNAi rice plants. *OsDof24-OX* plants were two weeks delayed in flowering time under SD condition and are also reduced in plant height. The obvious flowering late and reduced height phenotype indicates that OsDof24 is a repressor of rice heading date under 12 h light/12 h dark condition. In addition, the leaves grew in two dimensions instead of three dimensions. Thus, *OsDof24* is not only a regulator for heading date but also plays an important role in other developmental processes determining plant architecture. We also tried to study the effects of downregulating *OsDof24*, but we did not find an obvious phenotype which could be due to functional redundancy with other Dof genes or simply because the expression level was not downregulated sufficiently.

In rice there are two major flowering time pathways operational, one is controlled by *Hd1*, the other by *Ehd1* (Yano *et al.* 2000; Doi *et al.* 2004). In the *Hd1* pathway, Hd1 promotes rice flowering by activating expression of *Hd3a* under short day (SD) conditions (Yano *et al.* 2000). In the *Ehd1* pathway, *OsMADS51*, which is a B-type response regulator working upstream of Edh1, promotes flowering in the absence of a functional allele of *Hd1*. Ehd1 also promotes flowering by inducing expression of an *FT* like gene under SD condition (Doi *et al.* 2004). Although there are two different pathways, in the end, *Hd3a* and *RFT1* are most critical for the control of the heading date (Kojima *et al.* 2002; Tamaki *et al.* 2007; Komiya *et al*. 2008) and they are also recognized as candidate florigens (Komiya *et al.* 2009). In this study, we analyzed the expression of 11 flowering time genes in the *OsDof24-OX* lines in order to better understand the function of *OsDof24* in regulating flowering time. In the *OsDof24-OX* lines, *OsMADS50* was found up-regulated, whereas *OsMADS14*, *OsMADS1*, *RFT1* and *Hd3a* were down-regulated under SD condition, Expression of *Hd1*, *EHd1*, *OsMADS51*, *OsGI*, *OsMADS15* and *OsMAD18* was not significantly changed. Using bioinformatics we checked for presence of the Dof binding motif AAAG or its reverse complement sequence CTTT in the promoters of *OsMADS50*, *OsMADS1*, *OsMADS14*, *Hd3a* and *RFT1* after cloning them from *indica* rice. The Dof putative binding motif AAAG or its reverse complement sequence CTTT was found in all five promoters with a number varying from 14 to 22. This indicates that the OsDof24 protein might directly interact with the promoters and as a consequence regulate the expression. *OsMADS14* is a downstream gene of *Hd3a* and *RFT1* (Komiya *et al.* 2008). Therefore the downregulation of *Hd3a* and *RFT1* in transgenic plants overexpressing *OsDof24* leads to the downregulation of *OsMADS14*. Thus, overexpression of *OsDof24* specifically affects expression of sets of flowering time genes and it is likely that this takes place on the level of binding of OsDof24 protein to specific binding sites in downstream genes.

Based on previous reports and our results, we propose an updated model to explain the network in regulating heading date in rice (Figure 5). As explained above, heading date in rice is controlled by the *Hd1* and *Ehd1* pathways which share genes. Both *Hd1* and *Ehd1* are upstream of *Hd3a* and *RFT1* (Komiya *et al.* 2009) in the regulatory pathway controlling flowering time (Komiya *et al.* 2008). Hd3a and RFT1 promote flowering by inducing the expression of *OsMADS14* and *OsMADS15*, which interact with the protein OsMADS1 thereby promoting flowering (Lim *et al.* 2000). Normally, the expression of *RFT1* is very low compared to *Hd3a*, and Hd3a promotes rice flowering under SD condition. When *Hd3a* expression was down-regulated by an RNAi approach, *RFT1* became activated to regulate the flowering under SD condition (Komiya *et al.* 2008). Our results indicate that OsDof24 is an important regulator of flowering time by functioning as a repressor of flowering time genes including *Hd3a* and *RFT1*. Overexpression of *OsDof24* results in delayed flowering. *MADS50* was up-regulated in *OsDof24-OX* plants but it acts upstream of *FRT1* under LD (long day) condition (Lee *et al.* 2004; Komiya *et al.* 2009). Different molecular mechanisms controlling plant flowering time under different day length conditions would be the reason that over-expressing *OsDof24* did not promote flowering by up-regulating transcription of *OsMADS50* in the *OsDof24-OX* plants. Taken together, these results suggest that OsDof24 represses flowering by regulating expression of flowering time genes in particular via *Hd3a* and *RFT1* (Komiya *et al.* 2008). Understanding the molecular basis of the activation and repression of *Hd3a* and *RFT1* and the role of *OsDof24* therein will be very helpful for further investigate the mechanisms controlling rice heading date.

Materials and methods

Plants and growth conditions

Transgenic rice seedlings were pre-grown in plant developmental medium (basal ½ MS-medium with 10 g/L sucrose and 2.5 g/L phytagel, adjust ph to 5.8) before transfer to soil and grown under a dark-light cycle of 12h light (28°C, 80% humidity), and at 12 h darkness (21°C, 60% relative humidity) and at a light intensity (photosynthetically active radiation value) of 180 µmol/m2/s.

For protoplast isolation used in transient expression assays, rice seeds were sown into soil directly and grown in the same conditions as above. Flowering time was calculated from the germination to the day that the first flower opened. Plant height was measured from the soil to the top of the highest panicle 120 days after germination (DAG).

RNA isolation, cDNA cloning, RT-PCR and qPCR

Leaves from plants of hybrid rice 2186 (*Oryza sativa* L. ssp. *indica*) were ground in liquid nitrogen and total RNA was extracted using Trizol according to the manufacturer’s instructions (Invitrogen). Genomic DNA contaminants were removed from RNA samples by incubating with DNA-free™ (Ambion) at 37°C for 30 minutes. First-strand cDNA was synthesized starting from 1 μg of total RNA with SuperScript III reverse transcriptase (Invitrogen) as described by the manufacturer and used in RT-PCR and qPCRs as described below.

The ORF of *OsDof24* was obtained using PCR with primers *OsDof24*-F1 and *OsDof24*-R1 (Table S1a). PCR conditions were five minutes of initial denaturation at 98°C, 36 cycles of denaturation at 98°C for 30 s, annealing at 60°C for 30 s and extension at 72°C for 45 s, followed by a final extension step at 72°C for 10 min. To confirm the identity of the PCR products, they were first cloned in pCR-blunt II-TOPO vector (Invitrogen) and sequenced commercially (BaseClear, Leiden, The Netherlands).

For analysis of the expression profile of *OsDof24*, two week old seedling and flag leaves at 10 DAF, top second leaves, sheaths, stems, roots and panicles of rice cultivar Minghui 86 were used for RNA isolation and cDNA synthesis by qPCR. To determine the expression level of *OsDof24* and several heading date-related genes, flag leaves from the T_1 generation of *OsDof24*-OX and RNAi lines were used as material. Non-transgenic plants separated from the T_0 generation were used as control.

For qPCR assays, reactions were performed in an optical 96-well plate with an ABI PRISM® 7500 qPCR System (Applied Biosystems). SYBR® Green was used to monitor dsDNA synthesis. All reactions contained 12.5 µl 2× SYBR® Green Master Mix Reagent (Applied Biosystems), 2.0 ng cDNA and 10 pmol of each gene-specific primer in a final volume of 25 µl. Thermal cycling was as follows: 50°C for 2 min; 95°C for 10 min; 50 cycles of 95°C for 10 sec, 60°C for 30 sec, 72°C for 30 sec. Relative expression level of reporter and target genes was determined based on the $2^{-\Delta\Delta C}$T method (Livak and Schmittgen 2001). Both *Ubiquitin* and *Actin* were used as controls for cDNA quantification. Primers used for qPCR are listed in Table S1.

Sub-cellular localization of OsDof24 protein

In order to make a construct for subcellular localization studies, the ORF of *OsDof24* was PCR-amplified with primers *OsDof24*-F5 and *OsDof24*-R5 (Table S1a), and cloned as *Xhol*I/*Nco*I fragment into vector pTH2 (Chiu *et al.* 1996) between *Sal*I and *Nco*I. The resulting construct Pro35S::OsDof24-GFP carried the ORF of *OsDof24* in frame to the N-terminus of the green fluorescent protein (GFP) gene, was used in protoplast transformation. The empty vector pTH2 containing a *GFP* gene (with S65T mutation) driven by the CaMV 35S promoter was used as a control. Protoplast transformations were performed as described by Chen *et al.* (2006). After transformation, the protoplasts were incubated at 28°C overnight in K3 buffer and then observed using Confocal Laser Scanning Microscopy (CLSM) using excitation and emission filters Ex450-490 and BA520-560 for GFP.

Binary vector construction and rice transformation

For fusing the *OsDof24* promoter with the *GUS* gene, a 2,405 bp fragment upstream of the predicted translation start site was amplified from cultivar Minghui 86 using Phusion polymerase (Finnzyme) using primers OsDof24F and OsDof24R (Table S1d). The PCR fragment was subsequently cloned into vector pCR2.1 Topo (Invitrogen), sequence verified (BaseClear, Leiden, The Netherlands) and then inserted into vector pCAMBIA-1391Z (GenBank Accession AF234312) with *Eco*RI/*Nco*I sites, generating construct *ProOsDof24::GUS*.

For constructing an *OsDof24* overexpressing vector, the full length sequence of *OsDof24* was PCR-amplified with primers *OsDof24*-F2 and *OsDof24*-R2 (Table S1) and then digested with *Nco*I/*Bam*HI. The excised fragment was cloned using the same sites between the *GOS2* promoter and the *nos* terminator sequence in binary vector pCAMBIA-1300intC (GenBank Accession AF294978).

To down-regulate expression of *OsDof24* using an RNAi approach a fragment of the gene was obtained by PCR and then inserted into pHANNIBAL vector first (Wesley *et al.* 2001). The antisense fragment of *OsDof24* was generated using primers OsDof24-F3 and OsDof24-R3 (Table S1a), then digested by *Cla*I/*Bam*HI and finally inserted into pHANNIBAL using the same sites. The sense fragment of *OsDof24* was generated using primers *OsDof24*-F4 and *OsDof24*-R4 (Table S1a) and then excised with *Xho*I/*Kpn*I and finally inserted into pHANNIBAL carrying the antisense fragment. This plasmid was digested with *Sal*I/*Spe*I and the resulting fragment was inserted into vector pCAMBIA-1300intC cut with *Sal*I/*Xba*I. The resulting binary vector was used in rice transformation.

Rice transformation of *japonica* cultivar Zhonghua 11 was performed as described by Scarpella *et al*. (2000) instead that *A. tumefaciens* LBA4404 was used for transformation. Prior to growth in the greenhouse, transgenic seedlings were selected on a half-strength Murashige-Skoog medium supplied with 0.7% type I agarose (Sigma) and 25 µg/ml hygromycin. Transgenic lines were further characterised by Southern blotting using the *hpt* gene as a probe to determine copy number. Single copy lines were selected for further analysis.

Detection of GUS expression, cytological techniques and microscopy

Histochemical detection of GUS activity, cytological techniques and microscopy were performed as described earlier (Samach *et al.* 2000). Samples were viewed using a Leica MZ12 stereo microscope or a Leitz Diaplan microscope with bright-field optics settings and images were acquired with a Sony 3CCD Digital Photo Camera DKC-5000.

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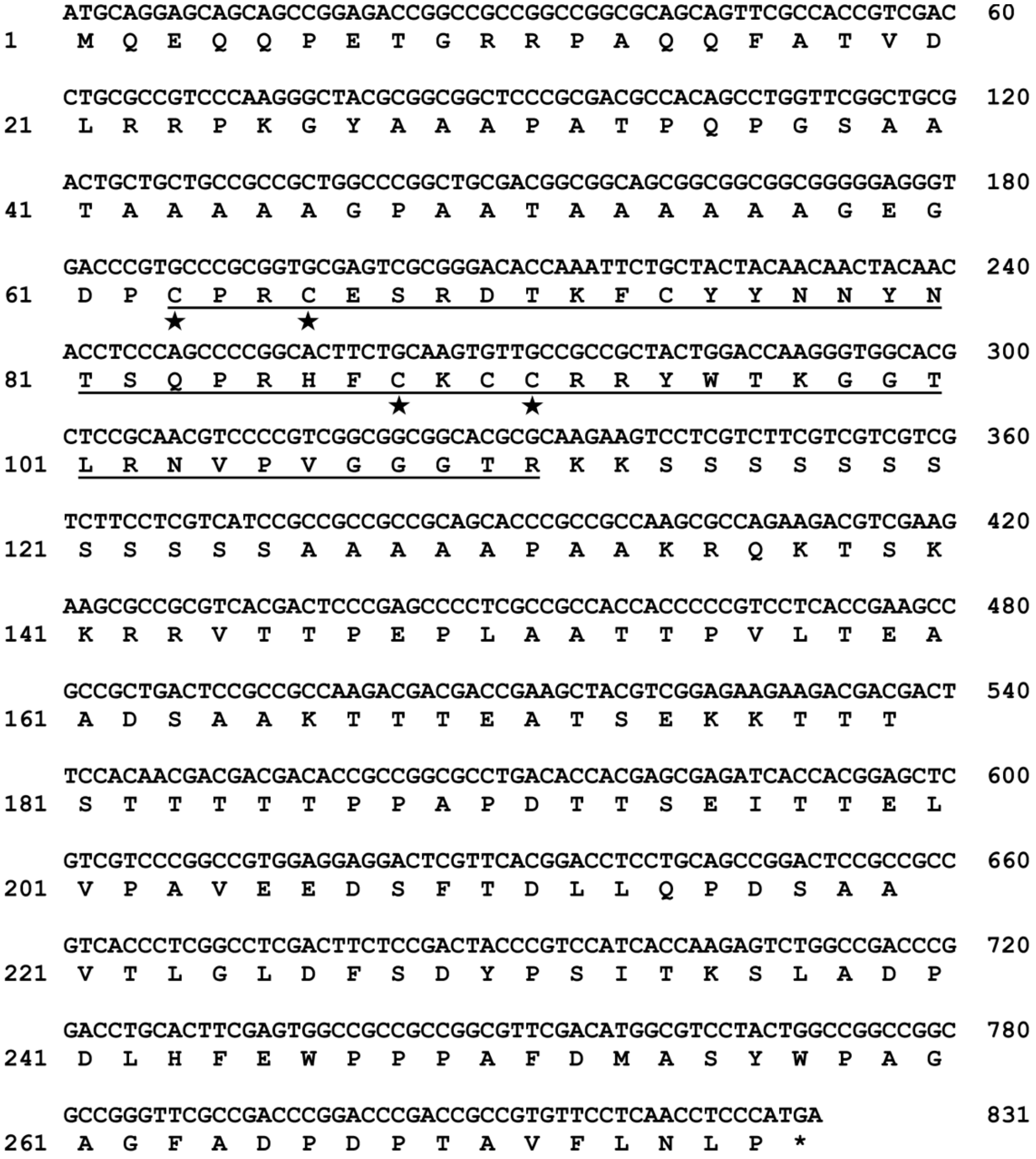
Supplemental data



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     CTGCGCCGTCCCAAGGGCTACGCGGCGGCTCCCGCGACGCCACAGCCTGGTTCGGCTGCG  120
21    L  R  R  P  K  G  Y  A  A  A  P  A  T  P  Q  P  G  S  A  A

     ACTGCTGCTGCCGCCGCTGGCCCGGCTGCGACGGCGGCAGCGGCGGCGGCGGGGGAGGGT  180
41    T  A  A  A  A  A  G  P  A  A  T  A  A  A  A  A  A  G  E  G

     GACCCGTGCCCGCGGTGCGAGTCGCGGGACACCAAATTCTGCTACTACAACAACTACAAC  240
61    D  P  C  P  R  C  E  S  R  D  T  K  F  C  Y  Y  N  N  Y  N
            ★        ★
     ACCTCCCAGCCCCGGCACTTCTGCAAGTGTTGCCGCCGCTACTGGACCAAGGGTGGCACG  300
81    T  S  Q  P  R  H  F  C  K  C  C  R  R  Y  W  T  K  G  G  T
                           ★        ★
     CTCCGCAACGTCCCCGTCGGCGGCGGCACGCGCAAGAAGTCCTCGTCTTCGTCGTCGTCG  360
101   L  R  N  V  P  V  G  G  G  T  R  K  K  S  S  S  S  S  S  S

     TCTTCCTCGTCATCCGCCGCCGCCGCAGCACCCGCCGCCAAGCGCCAGAAGACGTCGAAG  420
121   S  S  S  S  S  A  A  A  A  A  P  A  A  K  R  Q  K  T  S  K

     AAGCGCCGCGTCACGACTCCCGAGCCCCTCGCCGCCACCACCCCCGTCCTCACCGAAGCC  480
141   K  R  R  V  T  T  P  E  P  L  A  A  T  T  P  V  L  T  E  A

     GCCGCTGACTCCGCCGCCAAGACGACGACCGAAGCTACGTCGGAGAAGAAGACGACGACT  540
161   A  D  S  A  A  K  T  T  T  E  A  T  S  E  K  K  T  T  T

     TCCACAACGACGACGACACCGCCGGCGCCTGACACCACGAGCGAGATCACCACGGAGCTC  600
181   S  T  T  T  T  T  P  P  A  P  D  T  T  S  E  I  T  T  E  L

     GTCGTCCCGGCCGTGGAGGAGGACTCGTTCACGGACCTCCTGCAGCCGGACTCCGCCGCC  660
201   V  P  A  V  E  E  D  S  F  T  D  L  L  Q  P  D  S  A  A

     GTCACCCTCGGCCTCGACTTCTCCGACTACCCGTCCATCACCAAGAGTCTGGCCGACCCG  720
221   V  T  L  G  L  D  F  S  D  Y  P  S  I  T  K  S  L  A  D  P

     GACCTGCACTTCGAGTGGCCGCCGCCGGCGTTCGACATGGCGTCCTACTGGCCGGCCGGC  780
241   D  L  H  F  E  W  P  P  P  A  F  D  M  A  S  Y  W  P  A  G

     GCCGGGTTCGCCGACCCGGACCCGACCGCCGTGTTCCTCAACCTCCCATGA           831
261   A  G  F  A  D  P  D  P  T  A  V  F  L  N  L  P  *
```

Figure. S1 Nucleotide sequence and predicted amino acid sequence of the *OsDof24* gene (LOC_Os08g38220). The Dof domain in the N-terminus is indicated by underlines and the four cysteine residues which are characteristic for the Dof zinc finger of *OsDof24* are marked by stars.

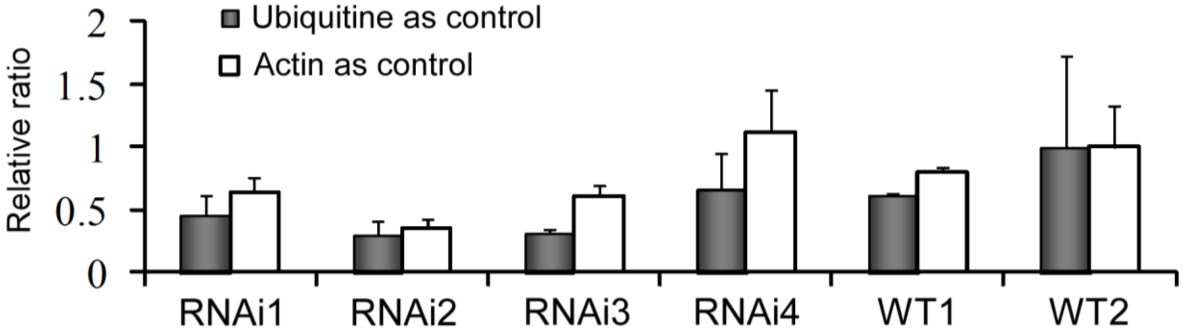

Figure. S2 Expression levels of *OsDof24* in the RNAi transgenic plants. *Ubiquitin* and *Actin* gene expression levels were used as internal controls for normalization of cDNA quantities. Bars represent means and standard deviations (n=3 independent qPCRs).

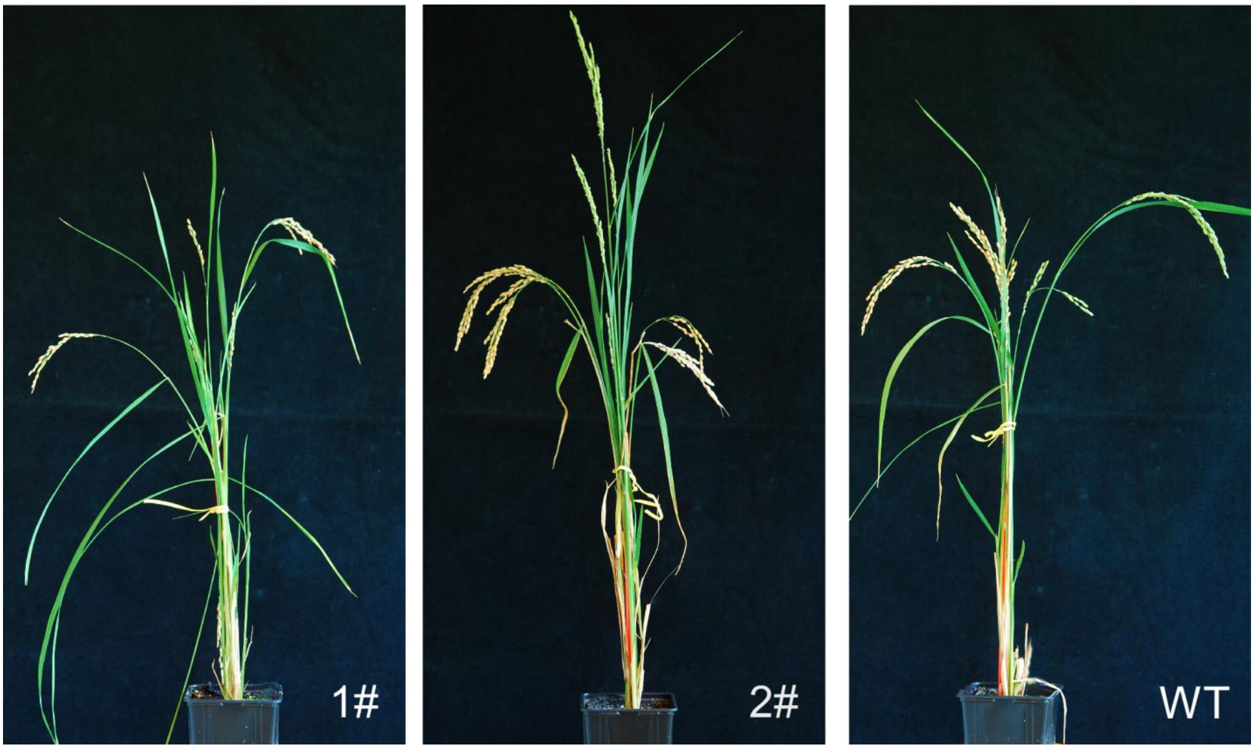

Figure. S3 Downregulation of *OsDof24* using transgenic RNAi lines 1 and 2 from Fig. S2 does not result in phenotypical changes. The results of two independent *OsDof24* RNAi transgenic plants and one control plant are shown.

Table S1 Primer sequences used for gene cloning, RT-PCR, real time quantitative PCR and cloning of the *OsDof24* promoter (LOC_Os08g38220).

Table S1a Primers used for *OsDof24* cDNA cloning and vector construction

Primer name	Primer Sequences (5' to 3')	Restriction enzyme site
OsDof24-F1	CGGGATCCATGCAGGAGCAGCAGCCGGA	*Bam*HI
OsDof24-R1	GGAATTCTCATGGGAGGTTGAGGAACACGGC	*Eco*RI
OsDof24-F2	CATGCCATGGAGGAGCAGCAGCCGGAG	*Nco*I
OsDof24-R2	CGGGATCCTCATGGGAGGTTGAGGAAC	*Bam*HI
OsDof24-F3	CCATCGATGCTGCCGCCGTCGCAG	*Cla*I
OsDof24-R3	CGGGATCCAGCAGCCGGAGACCG	*Bam*HI
OsDof24-F4	CCGCTCGAGAGCAGCCGGAGACC	*Xho*I
OsDof24-R4	GGGGTACCGCTGCCGCCGTCGCAG	*Kpn*I
OsDof24-F5	CCGCTCGAGATGCAGGAGCAGCAGCCGGAG	*Xho*I
OsDof24-R5	CATGCCATGGATGGGAGGTTGAGGAACACG	*Nco*I

Table S1b Primers used for RT-PCR

Gene name	Forward primer (5' to 3')	Reverse primer (5' to 3')
Hd3a	ATGGCCGGAAGTGGCAGGGAC	ATCGATCGGGATCATCGTTAG
Hd1	TTCTCCTCTCCAAAGATTCC	CATACGCCTTTCTTGTTTCA
Ehd1	GTTGCCAGTCATCTGCAGAA	GGATGTGGATCATGAGACAT
RFT1	CAAGGATCGTCTCCAATG	ACCAGCCAGTGTAGATAC
OsMADS51	GTTTGCTCTGCTCCTACTC	ACTCCTCCTCCAGCATTGAA
OsMADS14	TCCTATGCAGAAAAGGTCCTT	GGACGAAGCCAAAATATACAC
OsMADS15	CGTCGTCGGCCAAACAG	TGACTTCAATTCATTCAAGGTTGCT
OsMADS50	CAGGCCAGGAATAAGCTGGAT	TTAGGATGGTTTGGTGTCATTGC
OsGI	TGGAGAAAGGTTGTGGATGC	GATAGACGGCACTTCAGCAGAT
OsMADS18	CCAAACTGGATGCACTTCAG	ATCAATATCGCTGGAAGATG
OsMADS1	TCCATATGTCCTGGCAAGAT	AAGAGAGCACGCACGTACTT
Ubiquitin	AGCAGAAGCACAAGCACAAG	AAGCCTGCTGGTTGTAGACG

Table S1c Primers used for qPCR

Gene name	Forward primer (5' to 3')	Reverse primer (5' to 3')
OsDof24	ACGAGCGAGATCACCA	GGTCGGCCAGACTCTT
Hd3a	CACCTATGGCTCCAAGAC	GACCAACCAATGTAGATACTC
OsMADS14	CGGTTGCGAGACGAGGAA	GAAAGACGGTGCTGGACGAA
OsMADS50	CAGGCCAGGAATAAGCTGGAT	TTAGGATGGTTTGGTGTCATTGC
RFT1	CAAGGATCGTCTCCAATG	ACCAGCCAGTGTAGATAC
OsMADS1	TCCATATGTCCTGGCAAGAT	AAGAGAGCACGCACGTACTT
Ubiquitin	AGCAGAAGCACAAGCACAAG	AAGCCTGCTGGTTGTAGACG
Actin	GACCCAGATCATGTTTGAGACC	CATCACCAGAGTCCAACACAATAC

Table S1d Primers used for cloning of promoter constructs

Gene name	Forward primer (5' to 3')	Reverse primer (5' to 3')
OsMADS14	GGATCCAGAGCTATGATGGAGGCTCG	GAATTCCTTCCTCCTGTTCTTCCTCC
Hd3a	GGATCCGTAACATATTTGCCACCTATTG	CCATGGCGATCTTGCAAAAAACCCTG
OsMADS50	CGGGATCCGGAGTAGTACTTACTAGTGC	CATGCCATGGGAACCAACCAACCACACGAG
RTF1	CGGGATCCGCAAGTCGATCTGGAAGCTAG	CATGCCATGGGTCAAATTAATAACCTCTAAC
OsMADS1	CGGGATCCCAACACGAGGAAGAGGCAACAC	CATGCCATGGCTTCTTCCTCCTCCTCTC
OsDof24	CAGGTACTATAGCAGGCTATAAGGCCGC	CCATGGTGGCTGCTCTGGTCCCTCCGCGCG