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ORIGINAL PAPER

Heading date gene, *dth3* controlled late flowering in *O. Glaberrima* Steud. by down-regulating *Ehd1*

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Abstract Heading date in rice is an important agronomic trait controlled by several genes. In this study, flowering time of variety Dianjingyou 1 (DJY1) was earlier than a near-isogenic line (named NIL) carried chromosome segment from African rice on chromosome 3S, when grown in both long-day (LD) and short-day (SD) conditions. By analyzing a large F_2 population from NIL × DJY1, the locus *DTH3* (QTL for days to heading on chromosome 3) controlling early heading date in DJY1 was fine mapped to a 64-kb segment which contained only one annotated gene, a MIKC-type MADS-box protein. We detected a 6-bp deletion and a single base substitution in the C-domain by sequencing *DTH3* in DJY1 compared with *dth3* in NIL, and overexpression of *DTH3* caused early flowering in callus. Quantitative real-time PCR revealed that the transcript level of *dth3* in NIL was lower than that *DTH3* in DJY1 in both LD and SD conditions. The *Early heading date 1* (*Ehd1*) which promotes the *RFT1,* was up-regulated by *DTH3* in both LD and SD conditions. Based on Indel and dCAPs marker analysis, the *dth3* allele was only present in African rice accessions. A phylogenetic analysis based on microsatellite genotyping suggested that African rice had a close genetic relationship to *O. rufipogon* and *O. latifolia*, and was similar to *japonica* cultivars. *DTH3* affected flowering time and had no significant effect on the main agronomic traits.

Keywords Near-isogenic lines · Days to heading · Flowering regulator · African rice MADS-box

Abbreviations

DJY1	Dianjingyou 1
Ehd1	*Early heading date 1*
LD	Long-day
MAS	Marker-assisted selection
NILs	Near-isogenic lines
SD	Short-day
SSR	Simple sequence repeat

Introduction

African cultivated rice (*Oryza glaberrima*), which originated from *Oryza barthii* (formerly known as *Oryza* breviligulata) in the Niger River delta (Jones et al. 1997; Khush 1997), has tolerances to deep water, iron toxicity and infertile soils, and resistance to bacterial leaf blight (Linares 2002). As a valuable genetic resource *O. glaberrima* is not fully explored. The genetic control of heading date in African rice when grown in China is not well understood. In fact, heading date is one of the most

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important traits for adaptation of rice (*Oryza sativa* L.) to cultivation areas and crop seasons. Large variations in heading date occur among cultivated rice varieties. Heading date is determined by a combination of basic vegetative growth (BVG), photoperiod-sensitivity (PS) and temperature-sensitivity (TS), which are each controlled by genetic factors and environmental conditions (Chang et al. 1969; Hosoi 1981; Sato and Takahashi 1983). Quantitative trait loci (QTLs) contributing to heading date in *Oryza* species have been intensively explored and mapped. The duration of BVG was controlled by seven loci, including *Ef-1* on chromosome 10 (Tsai 1986), *Ef-3* on chromosome 2 (Yang et al. 2005) and *Ef-7* on chromosome 6 (Yuan et al. 2009). Most of the known heading date loci control PS. For example, *Hd1*, which is sensitive to day-length, is on chromosome 6 (Yano et al. 2000).

Regulatory mechanisms controlling flowering have been studied extensively in *Arabidopsis* (*Arabidopsis thaliana*) as a long-day (LD) model plant. In this LD species, flowering is promoted by the *CONSTANS* (*CO*) gene and regulated by the *GIGANTEA* (*GI*) gene, a circadian clock gene (Park et al. 1999; Sothern et al. 2002). *CO* activates expression of *FLOWERING LOCUS T* (*FT*) (Kardailsky et al. 1999; Kobayashi et al. 1999). *SUPPRESSOR OF OVEREXPRESSION OF CO1* (*SOC1*) is a flowering activator downstream of *CO* and integrates signals from multiple flowering pathways such as photoperiod, gibberellin and vernalization (Lee et al. 2000; Onouchi et al. 2000; Moon et al. 2003). In contrast to *Arabidopsis*, rice is a short-day (SD) plant whereby flowering is inhibited during early developmental stages, but induced when the day-length shortens in the fall. Although the roles of genes involved in the photoperiod pathway are generally conserved between rice and *Arabidopsis*, some differences exist. In rice, *OsGI* (a rice ortholog of *Arabidopsis GI*) inhibits flowering in LD conditions, and promotes flowering in SD conditions (Hayama et al. 2003). *Hd1*, a rice ortholog of *Arabidopsis CO*, is regulated by *OsGI* and promotes flowering in SD conditions (Yano et al. 2000). *Hd3a* is a homolog of *Arabidopsis FT*, and is positively regulated by *Hd1* in SD conditions (Kojima et al. 2002). *Hd1* represses flowering by down-regulating *Hd3a* expression in LD conditions (Kojima et al. 2002). *Hd3a* is also regulated by *Early heading date 1* (*Ehd1*), which encodes a B-type response regulator (Doi et al. 2004). Additionally, *RID1/OsID1/Ehd2*, which is homologous to maize (*Zea mays*) *Indeterminate 1* (*ID1*), is necessary for the expression of *Ehd1* regardless of photoperiod (Matsubara et al. 2008; Park et al. 2008; Wu et al. 2008). *Ghd7*, encoding a CCT domain protein, is an important regulator of heading date and yield potential in rice. It represses the expression of *Ehd1* and *Hd3a*, thereby delaying flowering in LD conditions (Xue et al. 2008). *DTH8/Ghd8*, encoding a putative *HAP3* subunit of the CCAAT-box-binding transcription factor, suppresses flowering and influences plant height and yield potential, and represses *Ehd1* in LD conditions (Wei et al. 2010; Yan et al. 2010).

Many flowering regulators, such as *RID1/OsID1/Ehd2, Ghd7, DTH8/Ghd8*, *OsCOL4, OsphyB* and others, play important roles in influencing many agronomic traits (Takano et al. 2005; Matsubara et al. 2008; Park et al. 2008; Wu et al. 2008; Lee et al. 2010). For example, plants with the *ehd2* mutation flower much later than wild type, and have more tillers and smaller spikelets (Matsubara et al. 2008). *Ghd7* and *DTH8/Ghd8* suppress flowering in LD conditions and influence plant height and yield (Xue et al. 2008; Wei et al. 2010; Yan et al. 2010). However, these genes are difficult to manipulate directly in rice breeding because their large effects lead to disharmony among heading date, plant height and yield.

We developed a NIL (Near-isogenic line) by MAS (molecular marker-assisted selection) and back-crossing to the *japonica* variety DJY1 (Dianjingyou 1), and the African rice accession IRGC102203 as the donor parent. Therefore, the objectives of this study were to elucidate the molecular basis of heading date differences between Asian and African rice, and we study the differentiation of *DTH3* (days to heading on chromosome 3 in DJY1) in Asian rice and African rice.

Materials and methods

Parental materials and mapping populations

The *japonica* variety DJY1 (Dianjingyou 1) is from Yunan Province, China, and IRGC102203 is an *O. glaberrima* accession. We developed a NIL by backcross and molecular marker-assisted selection (MAS) using DJY1 as the recurrent parent, and IRGC102203 as the donor. To assess the purity of the DJY1 background of NIL, we surveyed it using 192 SSR markers that were evenly distributed across the 12 chromosomes of rice (McCouch et al. 2002). Only one foreign chromosomal fragment of about two BACs was identified on chromosome 3 (Supplementary Fig. 1a, b). NIL and DJY1 were grown in Nanjing (day-length >14 h) and Hainan (day-length <11 h). For analysis of diurnal expression patterns of flowering genes, NIL and DJY1 were grown in LD (14 h light/10 h dark) and SD (10 h light/14 h dark) conditions for 45 days. Nipponbare, Nip (*hd1*), ZS (*ghd7*), Mh (*Ghd7*), Asominori and CSSL61 (*dth8*) were grown under LD conditions for 40 days.

Mapping the heading date gene

The inserted fragment in NIL is between markers g91 and Ins11. We surveyed heading dates of the NIL × DJY1 F_2

population ($n = 298$) in the summer of 2008 in Nanjing. In 2009, we planted a large F_2 population ($n = 18{,}000$) in Nanjing, selected 2,000 individuals with the latest heading dates, and 1,500 with the earliest heading dates for fine mapping following the approach described by Zhang et al. (1994). We also planted an F_2 population ($n = 10{,}000$) in Hainan (NSD) during the winter of 2009 for mapping. All materials were planted at a spacing of 16.5 cm × 16.5 cm.

DNA was extracted from fresh leaves of each plant following the method of Dellaporta et al. (1983). The PCR protocol was as described by Chen et al. (1997). PCR products were separated on an 8% non-denaturing polyacrylamide gel and detected using the silver staining method of Sanguinetti et al. (1994). To develop new SSR markers, an appropriate genomic sequence was obtained from the International Rice Genome Sequencing Project database (IRGSP; http://www.rgp.dna.affrc.go.jp/IRGSP/index.html1). Suitable microsatellite sequences were selected using on-line SSR searching software SSRIT (http://www.gramene.org/microsat/) and subjected to SSR primer design using Primer Premier 5.0 software. Additionally, insertion–deletion (InDel) markers were developed (insertions or deletions of 5 bp) by analyzing sequence differences between *japonica* variety Nipponbare and *indica* variety 93-11 in the delimited region using BLASTN (http://www.ncbi.nlm.nih.gov/BLAST/). PCR primers of products 100–300 bp in length were designed at about 10-kb intervals based on the sequence differences. We designed dCAPS markers with dCAPS Finder 2.0 on website http://helix.wustl.edu/dcaps/dcaps.html. The primers for mapping see Supplementary Table 1.

RNA extraction and QRT-PCR

RNA extraction and QRT-PCR total RNA extraction kits were used following the manufacturer's instructions (Beijing Dingguo Biotechnology Co. Ltd., http://www.dingguo.com). First strand cDNA was reverse transcribed from DNase I-treated RNA with oligo (dT) as the primer. Gene expression was measured by QRT-PCR using the Ubiquitin gene as an internal control. QRT-PCR for *Ubiquitin, DTH3, OsGI, Hd1, Hd3a, Ehd1, Ghd7, RID1, OsMADS51, DTH8, OsMADS56* and *RFT1* are listed in Supplementary Table 1. QRT-PCR was carried out in total volumes of 25 μL containing 2 μL of the reverse-transcribed product above, 0.2 μM of each primer and 13 SYBR green PCR master mix (TaKaRa Co. Ltd., http://www.takara.com.cn). The PCR was performed with a Bio-Rad iCycler (http://www.bio-rad.com/) using the following program: 95°C for 30 s, then 40 cycles of 95°C for 5 s, 60°C for 34 s. Changes in gene expression were calculated via the DDCT method (Livak and Schmittgen 2001).

Vector construction and transformation

The full-length coding region of *DTH3* was isolated by PCR with primer pair m1 from NIL and DJY1. The PCR product was subcloned into a pGE blunt end cloning vector. The cDNA was subcloned into pCUbi1390 binary vector using primer vector DTH3 with the restriction enzyme sites BamHI and SpeI (Supplementary Table 1). The resultant plasmid was introduced into NIL and DJY1 by means of *Agrobacterium*-mediated transformation (Hiei et al. 1994).

Statistical analyses

In the analysis of genetic relationships between African rice and different groups of cultivars, each polymorphism detected by 26 pairs of SSR markers was treated as a unit character by assigning a score of 1 or 0 to the presence or absence of a fragment (Supplementary Table 2). The genetic similarity coefficients among accessions assayed were estimated by genetic distance using Nei's (1987) unbiased genetic distance coefficient. The resulting genetic distance matrix was used for cluster analysis according to the unweighted pair-group method with arithmetic averages (UPGMA), using the software program NTSYSpc version (Rohlf 1992). To assess further the genetic relationships of African rice populations and selected rice varieties, a principle component analysis (PCA) was conducted based on the SSR variation patterns converted into the 1 and 0 matrixes. The correlation matrix was selected to calculate coefficients of the first three principal components, using MINITAB version 14.13 (Minitab Inc, State College, PA, USA). We used primers: F, 5′-TGGGGCATCGCTTGG CTAT-3′, R, 5′-CACCTGATTTGCTTCCCTTGA-3′ to detect the six-base deletion, and AACATGGATGTC-GAAACTGAGGAAT to detect the one-base substitution with restriction enzyme site EcoRI.

Results

Identification of flowering time in DJY1 and NIL

The flowering time of NIL was 7–10 days later than DJY1, whether grown in Hainan (natural short-day, NSD, day-length <11 h) or in Nanjing (natural long-day, NLD, day-length >14 h) (Fig. 1a, c). In artificial conditions (LD, 14 h light 10 h dark; and SD, 10 h light 14 h dark), NIL also flowered 7–10 days later than DJY1 (Fig. 1b). After heading, various traits, such as plant height, panicle and other agronomic traits, were not significantly different between NIL and DJY1 (Table 1; Fig. 1c, d, e).

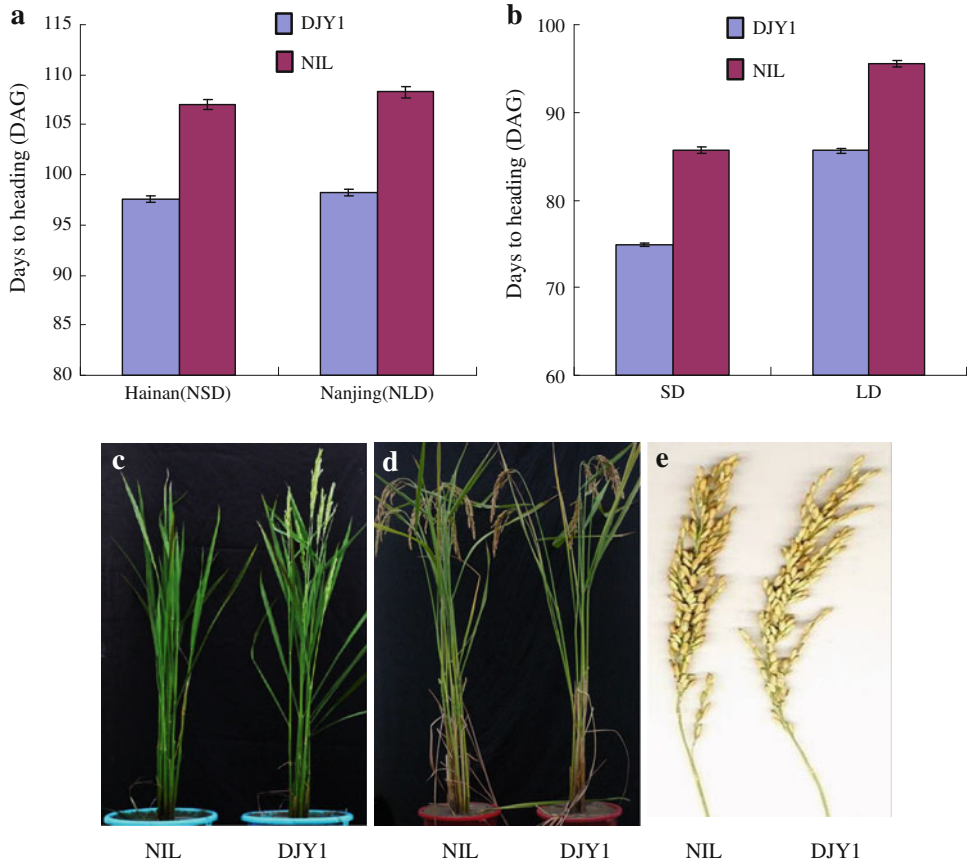

Fig. 1 **a** Comparisons of NIL and DJY1 heading dates in Nanjing (NLD) and Hainan (NSD); **b** days to heading for DJY1 and NIL under controlled SD (14 h light/10 h dark) and LD (10 h light/14 h dark); DAG, days after germination; **c**, **d** phenotypes of NIL and DJY1 grown under conditions at heading and maturity; **e** inflorescences of NIL and DJY1 grown under NSD

Table 1 Phenotypes of DJY1 and NIL under natural long-day conditions

The numbers of plant were more than 20

Lines	Plant height	Tillers	1,000-grain weight	Grains per spike
DJY1	107 ± 0.49	3.71 ± 0.12	28.79 ± 0.3	142.08 ± 1.57
NIL	108.2 ± 0.37	3.57 ± 0.13	29.32 ± 0.23	144.08 ± 1.75

Fine mapping and prediction of flowering gene

The inserted fragment containing *Hd9* in NIL was flanked by markers g91 and Ins11 on the short arm of chromosome 3 (Supplementary Fig. 1c). But our materials have the different phenotype, so we named *DTH3* (QTL for days to heading on chromosome 3) for early flower gene in DJY1 and *dth3* for late flower gene in NIL. The heading dates of the NIL × DJY1 F_2 population ($n = 298$) was surveyed in LD conditions, and we found that early heading plants ($n = 63$) possessed markers mainly from DJY1 (*DTH3*), and late heading genotypes ($n = 82$) had alleles mainly from NIL (*dth3*) (Fig. 2). We then planted a NIL × DJY1 F_2 population ($n = 18{,}000$) in Nanjing (NLD), selected 2,000 individuals with the latest heading dates, and 1,500 individuals with extremely early heading dates for fine mapping. The gene *DTH3* from DJY1 controlling early flowering was located between markers g71 and RM523, the distance between the two markers was 64 kb (Fig. 3), and the $F_{2:3}$ generation of recombinant individuals verified the fine mapping results (Table 2).

It was reported that *Hd3a* promoted heading under SD conditions whereas *Hd3b* caused late heading under LD and natural field conditions in Japan. The genetic distance between these two genes was about 1.6 cM (Monna et al. 2002). In our materials, NIL delayed flowering both in LD and SD conditions. In order to determine if separate genes controlled flowering under LD and SD conditions, we planted a NIL × DJY1 F_2 population ($n = 10{,}000$) in Hainan (NSD). The results suggested that the position mapped in both environments was the same. The genetic distances between markers g71 and RM523 in African rice (http://www.gramene.org/Oryza_glaberrima/Info/Index) and in Nipponbare (http://rgp.dna.affrc.go.jp/E/IRGSP/index.html) were 54 and 64 kb, respectively (Fig. 3). Based on candidate gene analysis by The Rice Annotation Project Database (http://rapdb.dna.affrc.go.jp/), we found a transcription factor, MIKC-type MADS-box gene, and

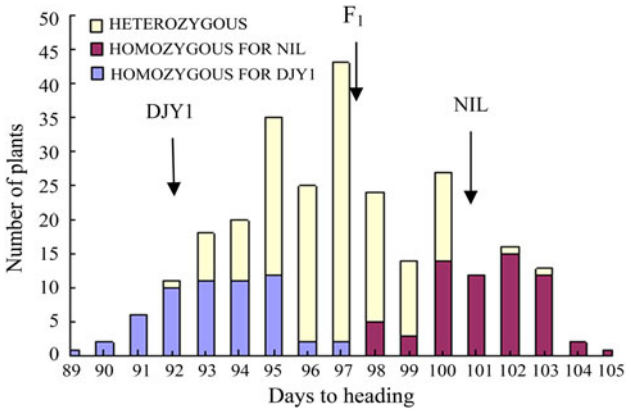

Fig. 2 Frequency distributions of days to heading in an F_2 population from NIL × DJY1. *Bars* indicate plants with three genotype classes: homozygous for NIL (*red*) and DJY1 (*blue*) alleles and heterozygous (*white*) for tightly linked SSR marker g71 and RM523 (color figure online)

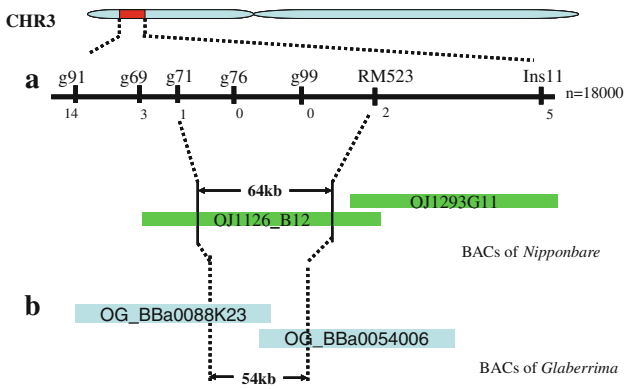

Fig. 3 High-resolution mapping of *DTH3*. **a** The genetic map of *DTH3* was based on recombination events among 18,000 F_2 plants from NIL × DJY1. The *numbers below markers* indicate the numbers of recombinants. The *wide horizontal lines* represent BAC/PAC clones of cv. *Nipponbare* with the accession numbers indicated. **b** The *wide horizontal lines* represent BAC/PAC clones of *O. glaberrima* with the accession numbers indicated

genome length of 29.7 kb. The cDNA sequence of this MADS-box gene have two differences between DJY1 and NIL at the code region. Six bases were inserted and base T instead of C occurred in the C-domain in NIL (Fig. 4). We considered the MADS-box gene as a good candidate for *DTH3*.

Overexpression of *DTH3* causes early flowering in callus

The transcript levels of *DTH3/dth3* gene in leaves of 45 day-old seedlings were measured. The expression level of *dth3* in NIL was lower than *DTH3* in DJY1, both in LD and SD conditions (Fig. 5a). To verify its function, *DTH3* was overexpressed in NIL and DJY1 driven by the maize ubiquitin (*ubi*) promoter (Fig. 6a). Overexpression of *DTH3* caused early flowering in callus of both DJY1 and NIL (Fig. 6b, c, d). The transcript level in the NIL callus with *Ubi*::*DTH3* was much higher than in NIL with the empty vector (Fig. 6e). Similar results were obtained by overexpression of the *dth3* in both NIL and DJY1 (date not show), suggesting that *DTH3* and *dth3* have the same function in both lines, but the expression level of *dth3* is weaker than *DTH3*. It is likely that *dth3* is a weaker allele of *DTH3*.

Expression patterns of *DTH3* in DJY1 and NIL

To determine whether the difference of expression patterns existed between DJY1 and NIL, mRNA level of *DTH3/dth3* was measured every 4 h in both LD (14L/10D) and SD (10L/14D). Transcript levels of *DTH3/dth3* changed over the 24-h cycle, the highest being at dawn and lowest at dusk in both LD and SD conditions and in both lines (Fig. 7a, b). However, the transcript levels of *dth3* were lower than *DTH3* over the 24-h cycle in both day-length environments. The *DTH3* transcript level in DJY1 gradually increased to a maximum at 9 weeks after germination, and then declined (Fig. 7c). The transcript level in NIL reached its maximum 1 week later than in DJY1. This apparently explained why the heading date of DJY1 was about 1 week earlier than NIL. Presumably, the accumulation of *DTH3* at the mRNA level plays an important role in promoting flowering.

Table 2 The key recombinant individuals for fine mapping from the F_2 population and results of their $F_{2:3}$ reconfirmations

Marker loci and genotypes						Days to heading											Estimated genotype
g91	g69	g71	RM523	bin22	Ins11	97	98	99	100	101	102	103	104	105	106	107	
H	H	H	H	D	D		1	2	5	6	1			1	1		H
N	N	N	N	N	H									3	1	3	N
H	H	N	N	N	N										9	7	N
H	H	H	N	N	N									7	5	9	N
D	D	D	H	H	H	8	8	1	3								D
N	H	H	H	H	H	3	3		6		1	1	2		2		H

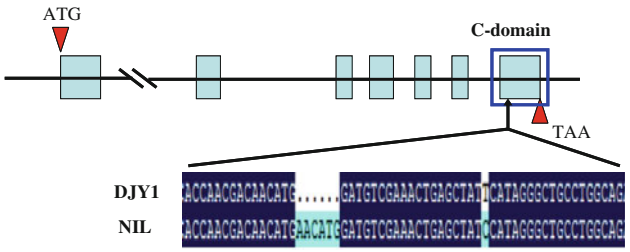

Fig. 4 Structure of *DTH3*. Seven exons (*filled boxes*) and six introns (*lines between the filled boxes*) are shown. There are two differences in the C-domain

Relationship of *DTH3* with other flowering time regulators

The expression of *dth3* was reduced in NIL relative to DJY1 in both LD and SD conditions. To investigate the relationship of *DTH3* with other flowering time regulators, the expression levels of *DTH3* and ten flowering-related genes (*Hd1, Hd3a, Ehd1, Ehd2, RFT1, OsMADS56, OsGI, DTH8, OsMADS51, Ghd7*) were analyzed in NIL and DJY1 grown in both environments (Fig. 5). *Hd1* was undetected both in LD and SD conditions over the 24-h cycle (data not shown). The mRNA levels of *DTH8* (Fig. 5d), *OsGI* (Fig. 5e), *OsMADS56* (Fig. 5f), *Ehd2* (Fig. 5g), *OsMADS51* (Fig. 5i) and *Ghd7* (Fig. 5j) did not differ between the two lines. However, the expression of *Ehd1* (Fig. 5b) and *RFT1* (Fig. 5c) in NIL were significantly lower than DJY1 in both environments, and *Hd3a* expression was reduced in LD, but unchanged in SD (Fig. 5h). These results indicate that *DTH3* accelerates rice flowering by up-regulating *Ehd1* which in turn activates *RFT1* both in LD and SD conditions, and up-regulates *Hd3a* only in LD. We further showed that the expression of *DTH3* was not influenced by *Hd1*, *Ghd7* and *DTH8* by examining *Hd1*, *Ghd7 a*nd *DTH8* NILs grown for 40 days under LD conditions (Fig. 8).

Differentiation of DTH3 in Asian rice, African rice and wild rice: molecular classification of related varieties

Pairs of Indel and dCAPs markers were designed from the 6-bp deletion and single base substitution in NIL. Two hundred and twenty Asian rice varieties, including 127 *indica* and 93 *japonica*, 22 wild rice and 53 African rice accessions were tested with these markers. All of the Asian cultivated rice (*indica* and *japonica*) accessions and wild rice lacked the six bases, whereas all African rice accessions contained them. All the Asian rice accessions and wild rice carried T at the polymorphic position, whereas both C and T were present in African rice (Table 3; Supplementary Table 3). It seems that during domestication of Asian rice, the six-base deletion and single nucleotide became fixed in Asian rice.

To understand the genetic relationship of African cultivated rice (*O. glaberrima*) with other rice groups we analyzed 26 pairs of SSR markers covering the entire rice genome. The dendrogram constructed from the results showed four groups, viz. *japonica*, *O. glaberrima*, *indica*, and wild rice. African rice was closely related to *O. rufipogon* and *O. latifolia*, and was more similar to *japonica* than to *indica* or other wild rice groups (Supplementary Fig. 2).

Discussion

The foreign fragment insert in NIL has the same position as *Hd9* in chromosome 3S (Lin et al. 2002, Supplementary Fig. 1c). The Nip (*hd9*) in which a small chromosomal segment of Kasalath including *Hd9* was substituted into the genetic background of Nipponbare. Nip *(hd9)* flowered later than Nipponbare in LD condition and was no difference in SD condition (Lin et al. 2002). The cDNA sequences of *DTH3* have single base substitution in Nipponbare and Kasalath (Supplementary Fig. 3). Interestingly, *OsMADS50*, reported by Ryu et al. (2009) as a LD-specific flowering activator was also present in our fine mapping region. It was likely that *Hd9*, *OsMADS50*, and *DTH3* are multiple alleles.

Rice is a SD plant. In SD conditions, signals from light and circadian clocks are received by *OsGI*, which regulates the expression of *Hd1* and *OsMADS51* (Izawa et al. 2003; Kim et al. 2007). *Hd1* up-regulates *Hd3a* expression (Kojima et al. 2002), while activating *OsMADS51* (Doi et al. 2004; Kim et al. 2007). In our study, *Hd1* was not detected in NIL and DJY1, and *Ehd2*, *OsMADS56*, *OsGI, DTH8*, *OsMADS51* and *Ghd7* were unchanged. *DTH3* up-regulated *Ehd1* and *RFT1* in both LD and SD conditions. It had been reported that *Ehd2/RID1/OsID1*, *OsPhyB*, and *OsCOL4* could up-regulate *Ehd1* in both situations (Takano et al. 2005; Matsubara et al. 2008; Park et al. 2008; Wu et al. 2008; Lee et al. 2010). But mutation or deletion of these genes not only influences heading date, but also agronomic traits, such as tiller number and 1,000-grain weight (Takano et al. 2005; Matsubara et al. 2008; Park et al. 2008; Wu et al. 2008; Lee et al. 2010). Thus these genes are difficult to use in rice breeding. While the phenotype in our materials, *dth3* delayed flowering both in LD and SD, and DJY1 and NIL are not very different in the main agronomic traits, so it was more useful in rice breeding and variety introduction without regard to the day-length.

Most flowering time regulators involved in photoperiod control are under circadian regulation. The mRNA level of *DTH3/dth3* showed a rhythm: high at dawn and low at dusk

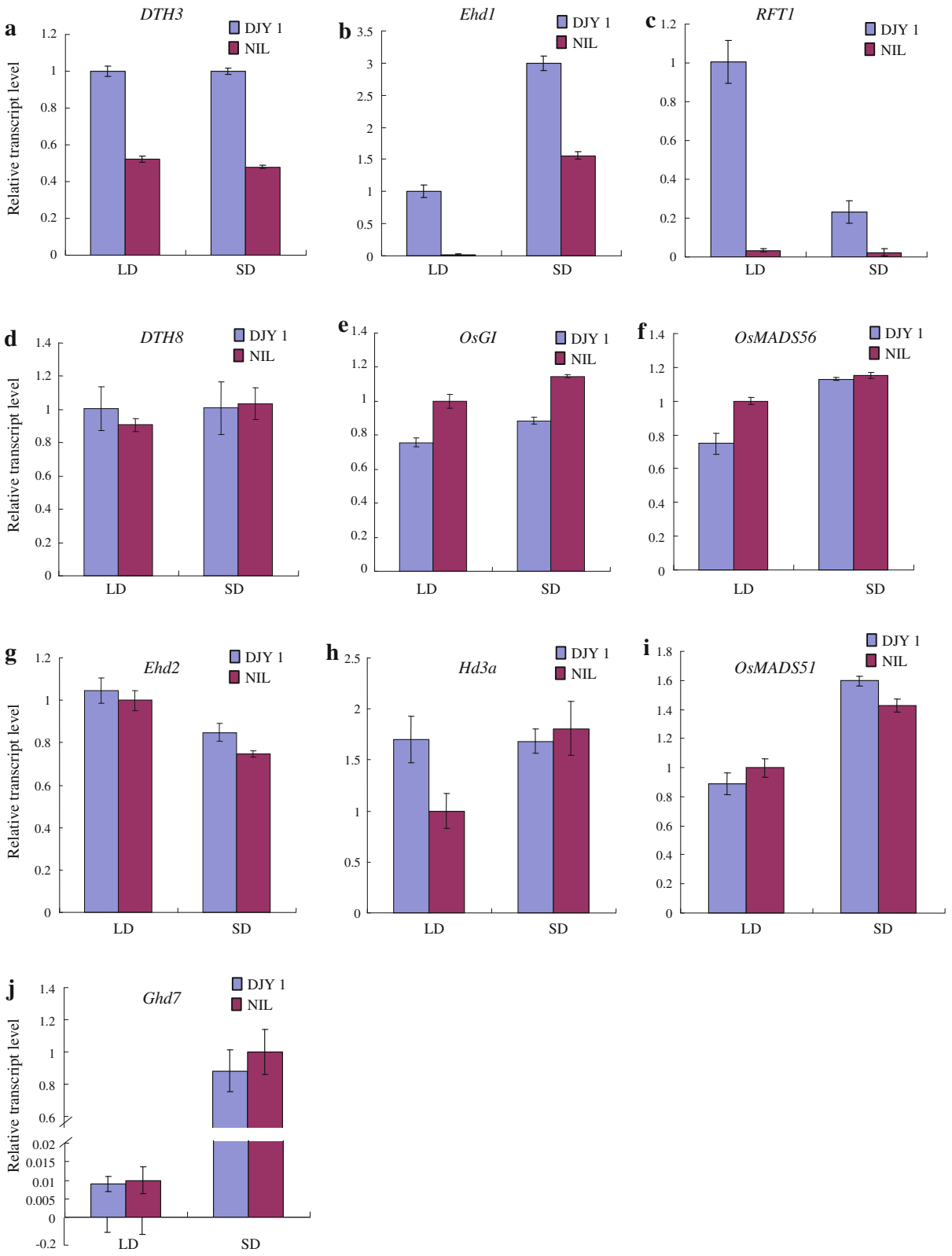

Fig. 5 Expression of *DTH3* and other flowering time genes in DJY1 and NIL. QRT-PCR was performed with total RNA from leaves of 45-day-old plants under SD and LD conditions. Samples were collected at the initiation of the light phase (ZT 0 h). Experiments were repeated at least

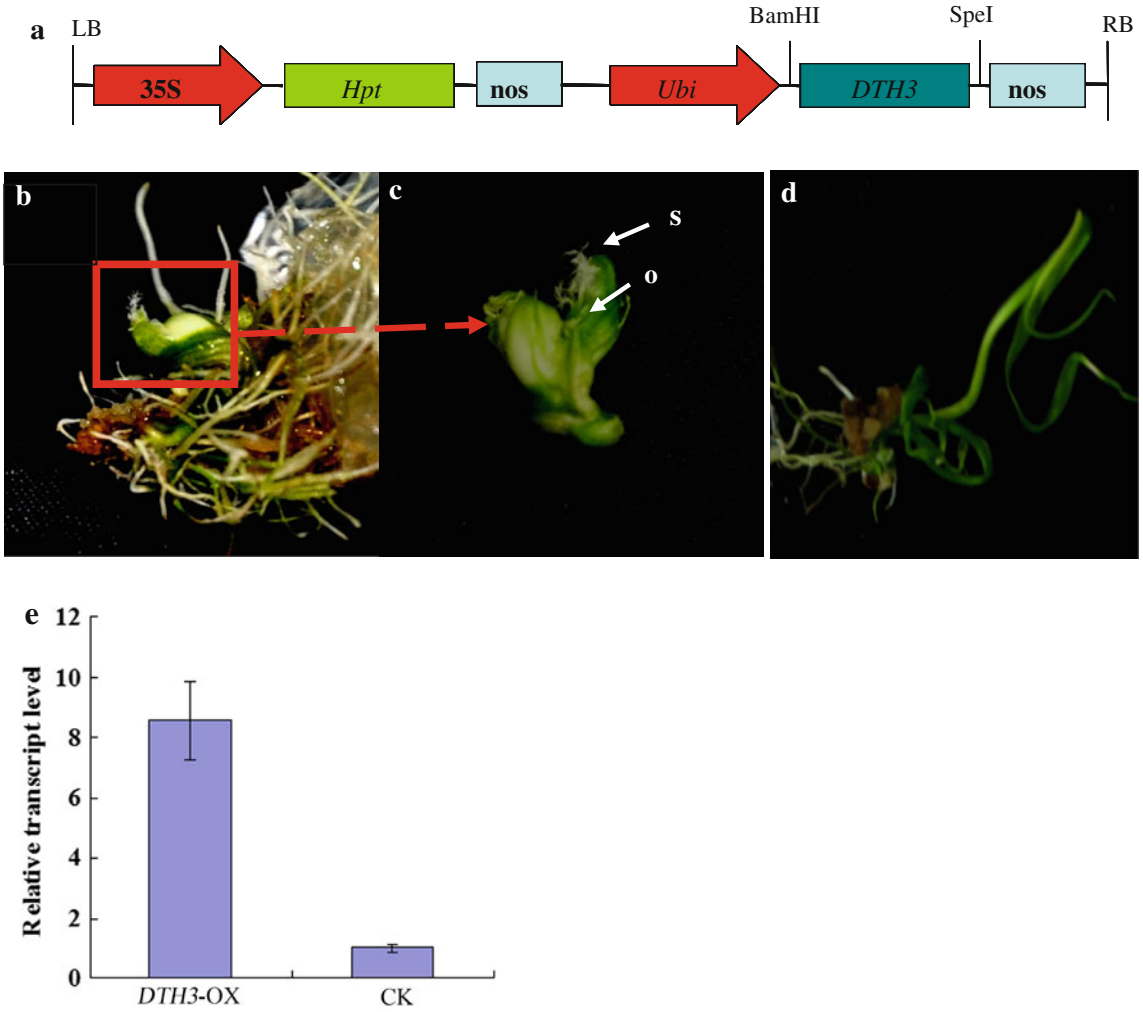

Fig. 6 Analyses of *DTH3* overexpressing plants. **a** Schematic diagram of *DTH3* construct. *Ubi* maize *ubi* promoter. **b** Overexpression *DTH3* in NIL; note flowering in callus. *s* stigma, *o* ovary. **c** Enlarge the *red frame* in **b**. **d** Callus with empty vector. **e** Quantitative real-time RT-PCR analyses of *DTH3* in callus, CK, NIL callus with empty vector (color figure online)

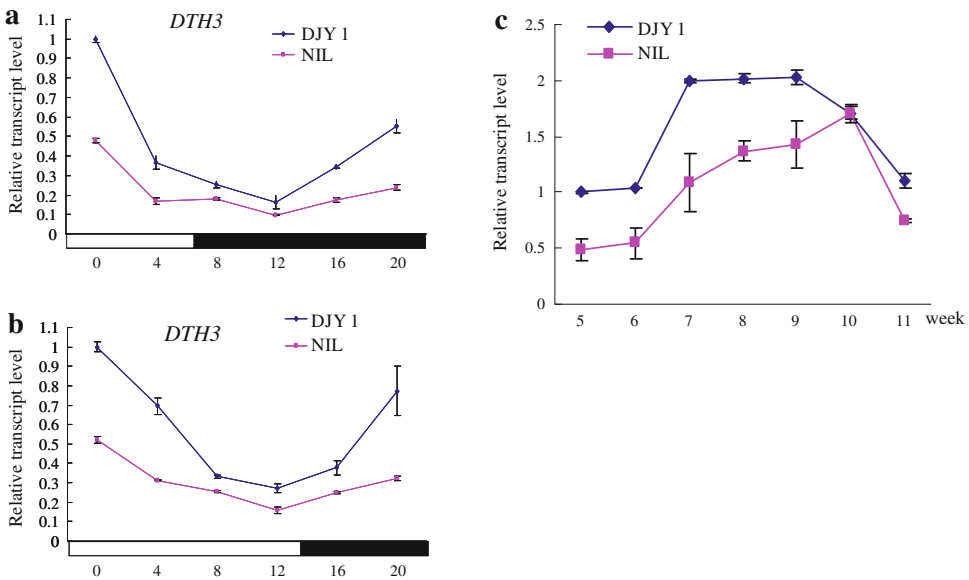

Fig. 7 Diurnal expression of *DTH3* in leaves under LD (**a**) and SD conditions (**b**) for 45 days. RNA were prepared from leaf blades at 4-h intervals over 24 h. c, Real-time RT-PCR analyses of *DTH3* at various developmental stages under LD conditions. RNA samples were collected from second leaf blades from the tops of plants grown under LD: 5, 6, 7, 8, 9, 10, 11 weeks after germination

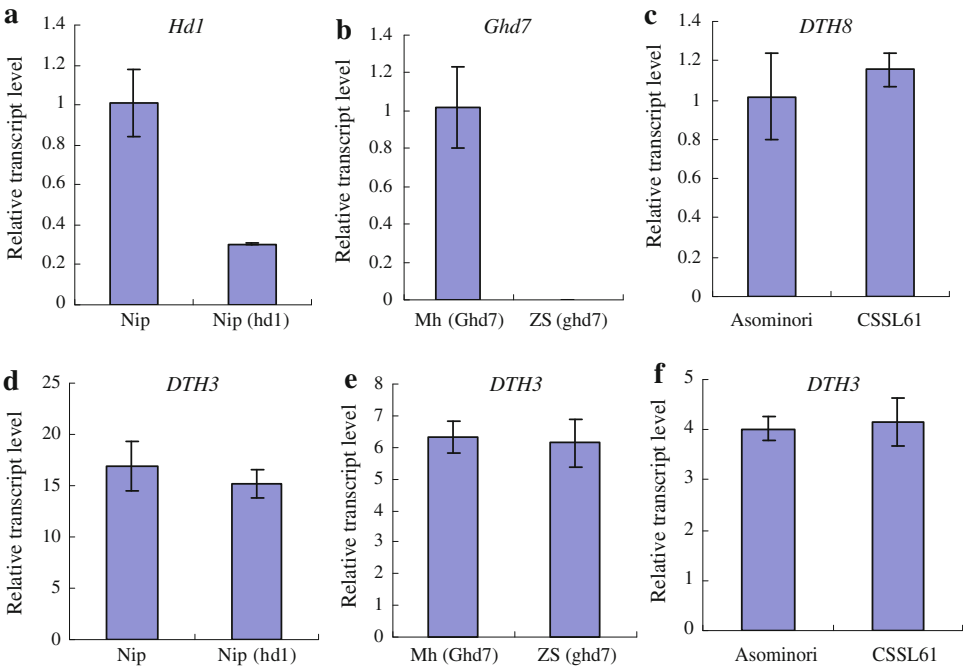

Fig. 8 **a** Expression in cv. Nipponbare (Nip) and Nip (*hd1*) was introgressed from cv. Kasalath in a cv. Nipponbare genetic background; **b** expression in Mh (*Ghd7*) introgressed from Minghui 63 and a NIL counterpart with the non-functional ZS (*ghd7*) allele from Zhenshan 97; **c** expression of *DTH8* in Asominori and a non-functional *dth8* allele from IR24 in CSSL61; **d** Expression of *DTH3* in Nip and Nip (*hd1*); **e** expression of *DTH3* in Mh (*Ghd7*) and ZS (*ghd7*); **f** expression of *DTH3* in Asominori and CSSL61. QRT-PCR was performed with total RNA from leaves of 40-day-old plants under LD conditions. Samples were collected at the initiation of the light phase (ZT 0 h). These experiments were repeated at least three times

Table 3 Analysis of *DTH3/dth3* in 22 wild species, 53 *O. glaberrima* strains, and 220 cultivars

a	C	T
japonica	0	93
indica	0	127
wild rice	0	22
O. glaberrima	26	27
b	AACATG	Delete
japonica	0	93
indica	0	127
wild rice	0	22
O. glaberrima	53	0

a and b, C→T and AACATG in *japonica*, *indica*, *wild rice* and *O. glaberrima*

both in LD and SD conditions, a similar pattern to *COL4 and RFT1* (Komiya et al. 2009; Lee et al. 2010). The transcript level in NIL was lower than in DJY1, and the transcript level in NIL reached its maximum 1 week later than in DJY1. This result was consistent with the observation that flowering time in NIL was about 1 week later than DJY1 in both LD and SD conditions. Recent reports suggested that the *SPL* (SQUAMOSA BINDING FACTOR-LIKE) family of transcription factors in *Arabidopsis* influences a series of phase transitions from juvenile to adult, as well as from vegetative to reproductive phase transitions, involved in age-related regulation of *SOC1* (Schwab et al. 2005; Wu and Poethig 2006; Wang et al. 2009). A MADS-box gene *SOC1* acts as an integrator in multiple flowering pathways involving photoperiod, gibberellin, aged-dependent regulation and vernalization in *Arabidopsis* (Lee et al. 2000; Onouchi et al. 2000; Moon et al. 2003). It is likely that *DTH3* is involved in age-dependent regulation or plays another role in the rice flowering pathway.

DTH3 encodes MIKC-type MADS-box proteins (Kaufmann et al. 2005), and has seven exons. MIKC-type MADS-box proteins contains four domains: MADS-domain (M) is required for DNA binding and dimerization (Schwarz-Sommer et al. 1990); The I domain, a less-conserved region, is also necessary for dimerization (Krizek and Meyerowitz 1996); the K domain plays an important role in the interaction between MIKC-type proteins (Fan et al. 1997; Yang and Jack 2004); and the C-domain is the least-conserved region, in some cases either possessing transactivation activity or contributing to the formation of multimeric complexes among MADS proteins (Cho et al. 1999; Honma and Goto 2001). In *Arabidopsis*, *SOC1* interacts directly with *AGL24*, a MADS-box transcription factor. This direct interaction confers a positive-feedback regulation of the expression of *AGL24* and *SOC1* to a quantitative threshold required for the transition from vegetative to reproductive growth (Liu et al. 2008). Recent research using ChIP analysis showed that *SOC1* directly binds to the modified CArG box in *LFY*. A missense mutation of *Arg24* resulted in the loss of *SOC1* binding to the *LFY's* promoter suggesting that *SOC1* does not work alone (Lee et al. 2008). *OsMADS56*

was a flower repressor under LD condition. Yeast-two hybrid was used to test the interaction between *DTH3/dth3* and *OsMADS56*; the result showed that *DTH3* can bind to *OsMADS56*, while *dth3* can not (Supplementary Fig. 4). It was further studied, whether *DTH3* and *OsMADS56* form complex to regulate heading date in rice. In our research, *dth3* had two mutations in the C-domain that may affect transcriptional activation. The C-terminal domain of *Arabidopsis thaliana AP1* (a member of the MADS superfamily) and its homologs perform a transcriptional activation function (Cho et al. 1999).

NIL contains a single DNA fragment from African rice in the background of *japonica* variety Dianjingyou 1 (DJY1). African rice is more resistant than Asian cultivars to diseases and pests, and superior in tolerance to fluctuations in water depth, iron toxicity, infertile soils and severe climatic conditions. Some *O. glaberrima* types also mature faster than Asian rice (Linares 2002). These characteristics are potentially useful for improving Asian rice. Here, we analyzed a flowering gene derived from African rice in NIL. Compared with *DTH3* in Asian rice, the *dth3* sequence in the segment from African rice was unique in possessing an additional 6-bp insert and in a single base substitution. African rice clustered in a group that was little differentiated from Asian rice and wild rice. African rice had a close genetic relationship to *O. rufipogon* and *O. latifolia*, and was more similar to *japonica* cultivars than to the other groups. The ancestor of African rice is *O. barthii*, and the ancestor of Asian rice is *O. rufipogon*; they presumably had a common ancestor before separation of the continents (Khush 1997). Our results showed that Asian rice and African rice had a common ancestor by using molecular markers (SSR, Indel and dCAPS) for analyzing genetic relationships between Asian rice and African rice. The two cultivated species were differentiated and domesticated in parallel in their respective geographical areas. *DTH3* was restricted to Asian rice and presumably differed from African rice as a result of selection. We also investigated the transcript level of *DTH3* in 43 cultivars including 20 *japonica* and 23 *indica* cultivars. The results showed that *DTH3* was not the main reason to affect heading date in Asian rice (Supplementary Fig. 5). To introduce the new favorable gene from other species of rice could broaden the genetic base, and it is beneficial for rice breeding.

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