SIP1 participates in regulation of flowering time in rice by recruiting OsTrx1 to Ehd1

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Summary

- Flowering (heading date) in rice (Oryza sativa) is an important agronomic trait that determines yield. The levels of histone H3 lysine 4 trimethylation (H3K4me3) modulated by TRITHORAX-like proteins regulate gene transcription, flowering time and environmental stress responses. However, plant TRITHORAX-like proteins have no known DNA-binding domain, and therefore the mechanism that gives sequence specificity to these proteins remains unclear.

- Here, we show that the rice TRITHORAX-like protein OsTrx1 is recruited to its target, Early heading date 1 (Ehd1), by the C2H2 zinc finger protein SDG723/OsTrx1/OsSET33 Interaction Protein 1 (SIP1).

- SIP1 binds to the promoter of Ehd1 and interacts with OsTrx1. Mutations in SIP1 led to a late heading date under long-day and short-day conditions. Defects in OsTrx1 or SIP1 led to reduced H3K4me3 levels at Ehd1, thus reducing Ehd1 expression.

- Together, our results show that the transcription factor SIP1 interacts with OsTrx1, allowing OsTrx1 to specifically target Ehd1, altering H3K4me3 levels, increasing Ehd1 expression and thereby promoting flowering.

Introduction

The timing of flowering, the transition from vegetative to reproductive development in plants, is determined by genetic pathways that integrate endogenous and environmental signals (Simpson & Dean, 2002; Izawa, 2007; Lee & An, 2015). Rice (Oryza sativa) flowering time (termed heading date) is an important agronomic trait for regional and seasonal adaptation, and heading at the proper time is a critical step for successful grain production (Izawa, 2007; Yeang, 2013; Sun et al., 2014). Rice has two homologs of Arabidopsis thaliana FLOWERING TIME LOCUS T (FT): Heading date 3α (Hd3α) and RICE FLOWERING TIME LOCUS T1 (RFT1). Hd3α and RFT1 encode proteins that function as floragens under short-day (SD) and long-day (LD) conditions, respectively (Yano et al., 2001; Kojima et al., 2002; Tamaki et al., 2007; Komiya et al., 2008, 2009). Hd3α and RFT1 are generated in the leaf phloem and transferred to the shoot apical meristem (SAM), where they induce reproductive development (Komiya et al., 2008; Hagiwara et al., 2009; Lee & An, 2015).

The positive and negative regulators of the florigen genes Hd3α and RFT1 form a network that regulates flowering in rice (Komiya et al., 2009). Upstream of Hd3α and RFT1, rice has a conserved flowering pathway, the Heading date 1 (Hd1) pathway, which is controlled by photoperiod (Komiya et al., 2008; Takahashi et al., 2009). Hd1, a homolog of CONSTANS (CO), promotes flowering under SD by the activation of Hd3α and represses flowering under LD by the suppression of Hd3α (Yano et al., 2000; Ishikawa et al., 2005, 2011), respectively. In addition to the Hd1 pathway, rice has a unique, independent flowering time pathway mediated by Early heading date 1 (Ehd1). Ehd1 encodes a B-type response regulator that promotes flowering by controlling the expression of FT-like genes under SD and LD (Doi et al., 2004). Ehd1, a central integrator involved in flowering time, is activated by Ehd2/OsID1 (OsINDETERMINATE 1/RID1 (Rice INDETERMINATE 1)) and Ehd4 under LD and SD conditions (Matsubara et al., 2008; Park et al., 2008; Gao et al., 2013). OsMADS50 promotes Ehd1 by suppressing the expression of (LEAFY COTYLEDON 2 and FUSCA 3-LIKE 1) OsLFL1, which encodes an inhibitor of Ehd1 under LD conditions (Lee et al., 2004; Ryu et al., 2009). OsMADS56 interacts with OsMADS50 in vitro, these two MADS-box proteins form a heterodimer and function antagonistically through the OsLFL1–Ehd1 pathway under LD (Ryu et al., 2009). OsMADS51, another MADS-box gene, promotes Ehd1...
expression under SD (Kim et al., 2007). In addition to being activated by MADS-box proteins, Ehd1 is suppressed by Grain Number Plant Height and Heading date7 (Ghd7), which encodes a CCT domain protein (Xue et al., 2008). Ghd7 is activated by EL1 (Early flowering 1)/Hd16, which encodes a casein kinase I protein (Dai & Xue, 2010), and is suppressed by Ehd3 (Matsubara et al., 2011).

In eukaryotic cells, dynamic changes in chromatin structure mediated by covalent modifications of DNA or histones frequently correlate with changes in transcription. The methylation of histone lysines, including histone H3 lysine 4 (H3K4), H3K9, H3K27, H3K36, H3K79 and H4K20, plays a crucial role in the regulation of key biological processes, such as cell cycle progression, transcription and DNA repair (Zhang & Reinberg, 2001; Liu et al., 2010). Except for H3K79 methylation, which is catalyzed by Dot1 family proteins, all other histone lysine methylations are carried out by SET (Su (var), Enhancer of Zeste and Trithorax) domain-containing enzymes (Takahashi et al., 2011). The SET domain family contains yeast Set1 and its orthologs in multiple biological processes, such as flowering time regulation (Bernstein et al., 2002; Santos-Rosa et al., 2008). Flowering promoted by OsTrx1/SDG723/OsSET33, a rice TRITHORAX-like protein, interacts with Ehd3 to repress the transcription of Ghd7 (Choi et al., 2014). The PHD motif of OsTrx1 binds the histone H3, allowing OsTrx1 to modify chromatin (Choi et al., 2014). However, the molecular mechanism of how OsTrx1 targets the flowering time gene and establishes H3K4me3 remains unclear. Here, we report that OsTrx1 interacts with the transcription factor Sip1, which allows OsTrx1 to bind to Ehd1. Defects in OsTrx1 and Sip1 result in late heading under LD and SD conditions and both act in the same flowering pathway in rice.

Materials and Methods

Materials

The authorities for all of the species under investigation include: University of Science & Technology of China and Anhui Agricultural University. The plants used in the study are in the Oryza sativa ssp. japonica cv Nipponbare background. All plants were regenerated from callus as described previously (Xu et al., 2015, 2016). T1 plants were generated from the seeds of self-pollinated T0 plants. For mutants generated by CRISPR/Cas9, the CRISPR/Cas9 constructs were segregated out in self-pollinated lines. All rice plants were grown in fields from 20 April to the beginning of October at Hefei (Anhui) in 2016 and 2017, and from 20 November to the beginning of February at Lingshui (Hainan), China in 2016 and 2017. The details of day length for rice growth in Hefei and Lingshui are described in Supporting Information Table S1. All plants were re-grown in a growth room at 26°C under a restricted LD photoperiod (14 h : 10 h, light : dark cycle) or a restricted SD photoperiod (10 h : 14 h, light : dark cycle). Arabidopsis thaliana ecotype WS plants were grown at 22°C under an LD photoperiod (16 h : 8 h, light : dark cycle). Seeds from the atxl-1 mutant have been described previously (Alvarez-Venegas et al., 2003; Ding et al., 2011a,b).
Plasmid constructs

The plasmids were constructed using the DNA primers and protocols described in Table S2. All cloned DNA was confirmed by DNA sequencing.

Yeast one-hybrid assay

Yeast one-hybrid assay was performed following the ‘Matchmaker Gold Yeast One-Hybrid Library Screening System User Manual’ (Clontech, Mountain View, CA, USA). Briefly, the different parts of the Ehd1 promoter were cloned into the pAbAi vector and SIP1 was fused with pGADT7 (AD-SIP1). Then, the proEhd1-pAbAi vectors were transferred into the yeast strain and grown on synthetic defined medium lacking uracil (Ura). The minimal inhibitory concentration of Aureobasidin A (AbA) for the bait strain was tested on medium lacking Ura. Then, AD-SIP was transferred into bait strains containing different proEhd1-pAbAi vectors. The protein–DNA interactions were examined on medium lacking leucine (Leu) and Ura with 250 ng ml⁻¹ AbA.

Yeast two-hybrid assay

The yeast two-hybrid assay was performed according to the manufacturer’s protocol (Clontech, user’s manual 630489). Briefly, the Saccharomyces cerevisiae strain AH109 was transformed with the bait construct pGBK7-OsTrx1, and then transformed with the cDNA library generated from rice leaves by Clontech. pGBK7-OsTrx1, pGBK7-OsTrx1N or pGBK7-OsTrx1C was then co-transformed with pGADT7-SIP1. Vectors lacking coding region insertions were used as negative controls. The yeast was scored for protein interaction based on its ability to grow on synthetic defined medium lacking tryptophan (Trp), Leu, histidine (His) and adenine. The primers used to generate the constructs are shown in Table S2.

Protein pull-down assays, co-immunoprecipitation (Co-IP) and immunoblot assays

For the pull-down assays, beads were incubated with 3 μg of fusion protein, washed and incubated with 3 μg of soluble protein overnight at 4°C. Mock controls included extracts prepared from either the His-Tag or glutathione transferase (GST) vectors. The beads were washed five times with a solution containing 20 mM Tris (pH 7.4), 150 mM NaCl and 0.05% Tween 20, separated on a sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel and analyzed by immunoblotting using an anti-GST antibody (GenScript, Nanjing, China; A00866-100, lot: 13D000626) or an anti-His antibody (Ambart, Shanghai, China; M30111M, lot: 273884).

For Co-IP, SIP1 was fused with FLAG tag and OsTrx1 was fused with HA tag, and cloned into the pUC19 vector. Co-IP was performed as described previously (Lu et al., 2017). Briefly, 1 × 10⁶ rice protoplasts were lysed with PEN-140 buffer (140 mM NaCl, 2.7 mM KCl, 25 mM Na₂HPO₄, 1.5 mM KH₂PO₄, 0.01 mM EDTA and 0.05% CA-630). The supernatant was pre-cleared with Protein G and precipitated with anti-HA (Sigma-Aldrich; H9658, lot: 095M4778V) antibodies. The protein complexes were isolated by binding to Protein G beads, followed by five washes with PEN-400 buffer (400 mM NaCl, 2.7 mM KCl, 25 mM Na₂HPO₄, 1.5 mM KH₂PO₄, 0.01 mM EDTA and 0.05% CA-630). The samples were analyzed by immunoblotting using anti-FLAG (Sigma-Aldrich; H6908, lot: SLBQ7119V) and anti-HA antibodies.

Complementation of the ostrx-2 and atx1 mutants

For ostrx1-2 complementation, the ProO Peterson:FLAG-OsTrx1 construct was generated by fusing the full-length cDNA of OsTrx1 to FLAG, and then inserting the fusion into a pCambia1300 vector harboring the 3100-bp OsTrx1 promoter. The construct was introduced into the Agrobacterium tumefaciens strain EHA105 and plants were regenerated from callus as described previously (Xu et al., 2015).

For atx1 complementation, the Pro355:RFP-OsTrx1 construct was generated by fusing the full-length cDNA of OsTrx1 to RFP, and then inserting the fusion into a pCambia1301 vector. The construct was introduced into the Agrobacterium tumefaciens strain EHA105 and transformed the atx1 mutant.

Generation of ostrx1, edh1 and sip1 mutants using CRISPR/Cas9

The CRISPR/Cas9 mutagenesis was performed as described previously (Xu et al., 2015, 2016). Briefly, the oligonucleotides used for OsTrx1, Ehd1 and Sip1 mutagenesis were designed with the help of CRISPR-PLANT tools (Yan et al., 2015). The oligonucleotides were inserted into the CRISPR/Cas9 vector pHUN4c12 with BalI. The binary constructs were then introduced into the Agrobacterium tumefaciens strain EHA105 and plants were regenerated from callus. The mutants were further confirmed by sequencing. The oligonucleotides are listed in Table S2.

Electrophoretic mobility shift assay

The electrophoretic mobility shift assay (EMSA) was performed as described previously (Su et al., 2017). Briefly, 1–2 μg of purified protein in binding buffer (10 mM Tris-HCl, pH 7.5, 100 μM KCl, 1 mM EDTA, 100 μg ml⁻¹ bovine serum albumin (BSA), 100 μM ZnCl₂, 6% glycerol, 1 mM diethiothreitol (DTT)) was mixed with 4 pmol γ⁻³²P ATP-labeled probe with or without various dosages of unlabeled probe at 4°C for 1 h. After separation in a 4.5% native non-denaturing acrylamide gel, the gel was exposed to X-ray film overnight. The sequence of the probe is shown in Table S2.

Transient expression in rice protoplasts and bimolecular fluorescence complementation

For bimolecular fluorescence complementation (BiFC), SIP1 and OsTrx1 were cloned into pUC-SPYCE (amino acids 156–239)
or pUC-SPYNE (amino acids 1–155). Rice protoplast isolation and transformation were performed as described previously (Zhang et al., 2011). Briefly, 7–10-d-old rice seedlings were cultured at 26°C on half-strength Murashige and Skoog (MS) medium under a 12 h : 12 h, light : dark cycle. Stem and sheath tissues were cut into 0.5-mm strips and immediately transferred into 0.6 M mannitol. After enzymatic digestion, W5 solution (154 mM NaCl, 125 mM CaCl₂, 5 mM KCl and 2 mM MES at pH 5.7) was added to the sample, followed by resuspension in MMG solution (0.4 M mannitol, 15 mM MgCl₂ and 4 mM MES, pH 5.7). The protoplasts were co-transformed with the corresponding constructs and examined under a confocal laser scanning microscope (Zeiss, LSM710) or immunoprecipitated with specific antibodies.

Promoter β-glucuronidase (GUS) assay

The vectors containing GUS driven by different promoters were transformed into rice protoplasts with or without SIP1. After incubation at 25°C for 12 h, the protoplasts were lysed with lysis buffer (25 mM Tris-HCl, pH 7.8, 1 mM DTT, 10% glycerol and 1% Triton X-100). After centrifugation, 100 µl of supernatant was mixed with 900 µl of fluorescent β-galactosidase (MUG) substrate mix (10 mM Tris-HCl, pH 8.0, containing 1 mM MUG and 2 mM MgCl₂). After incubation at 37°C for 30 min, the reaction was stopped with 40 mM Na₃CO₃. GUS activity was measured using a fluorometer (HITACHI, U-281; Thermo Fisher, Fluoroskan Ascent FL, Waltham, MA, USA) with 365 nm excitation wavelength and 456 nm emission wavelength. The luciferase (LUC) activity was measured using the GloMax 96 Luminometer system (Promega, E6501) with LUC mix (Promega, E1980).

Antibody generation

To generate Anti-OsTrx1, the N-terminus of OsTrx1 (amino acids 1–300) was expressed and purified. The antibody was generated by injection of a rat and conducted by Abmart.

To generate anti-SIP1, the N-terminus of SIP1 (amino acids 1–100) was expressed and purified. The antibody was generated by injection of rabbits and conducted by GenScript.

Reverse transcription and qPCR (quantitative real-time PCR)

Total RNA was isolated from the leaves of 80-d-old plants under LD conditions or from those of 45-d-old plants under SD conditions, and reverse transcribed with oligo(dT) primers (Promega); the amounts of individual gene transcripts were measured with gene-specific primers. RT-PCR analysis was performed with a CFX real-time PCR instrument (Bio-Rad) and SYBR Green mixture (Roche). The relative expression of the genes was quantified with the 2^(-ΔΔCT) calculation, using UBIQUITIN as the reference housekeeping gene for chromatin immunoprecipitation (ChIP) assays (see Table S2).

ChIP assay

ChIP assay was performed as described previously (Lu et al., 2017; Su et al., 2017). Briefly, 3 g of 80-d-old plants was ground and fixed with 1% formaldehyde for 10 min and quenched in 0.125 M glycine. The grounds were extracted in buffer I (0.4 M sucrose, 10 mM Tris (pH 8.0), 5 mM β-mercaptoethanol, 0.1 mM phenylmethylsulfonylfluoride (PMSF) and protease inhibitor cocktail) and filtered through Miracloth. After centrifugation, the pellet was extracted with buffer II (0.25 M sucrose, 10 mM Tris (pH 8.0), 10 mM MgCl₂, 1% Triton X-100, 5 mM β-mercaptoethanol, 0.1 mM PMSF and protease inhibitor cocktail) and then with buffer III (1.7 M sucrose, 10 mM Tris (pH 8.0), 10 mM MgCl₂, 1% Triton X-100, 5 mM β-mercaptoethanol, 0.1 mM PMSF and protease inhibitor cocktail). The nuclei were then lysed in lysis buffer (50 mM Tris, pH 8.0, 10 mM EDTA, 1% SDS, 5 mM β-mercaptoethanol, 0.1 mM PMSF and protease inhibitor cocktail) and the extract was sonicated to fragment the DNA to a size range of 300–500 bp. After centrifugation, the supernatant was diluted using dilution buffer (1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris (pH 8.0), 167 mM NaCl, 0.1 mM PMSF and protease inhibitor cocktail) and pre-cleared with protein A or protein G magnetic beads.

The specific antibodies anti-H3K4me3 (Abcam, Cambridge, UK; ab8580, lot: GR273043-6) and anti-FLAG (Sigma-Aldrich; F3165, lot: SLBQ7119V), or control IgG serum, were added to the pre-cleared supernatants for an overnight incubation at 4°C. The antibody–protein complexes were isolated by binding to protein A or protein G beads. The washed beads were heated at 65°C for 8 h with proteinase K to reverse the formaldehyde cross-linking and digest the proteins. The sample was then extracted with phenol/chloroform and the DNA was precipitated in ethanol and resuspended in water. The purified DNA was analyzed by RT-PCR with the gene-specific primers shown in Table S2.

Accession numbers

Sequence data from this article can be found in the GenBank/EMBL data libraries under the following accession numbers: OsTrx1/SDG723 (Os09g0134500) and SIP1 (NC_029264).

Results

OsTrx1 rescues the developmental defects caused by the atx1 mutation

As rice OsTrx1 is closely related to Arabidopsis ATX1, with 56% sequence identity and 70% similarity, we investigated whether OsTrx1 and ATX1 have similar functions. We generated a vector harboring a full-length cDNA from OsTrx1 fused with RFP driven by the Cauliflower mosaic virus (CaMV) 35S promoter (Pro35S:RFP-OsTrx1), which we transformed into the Arabidopsis...
axl-1 mutant. The early flowering and dwarf phenotypes caused by the axl mutation were rescued by OsTrx1 (Fig. S1), indicating that OsTrx1 might function as an H3K4 methyltransferase during rice development.

OsTrx1 is involved in the Ehd1–RFT1 pathway

To investigate the roles of OsTrx1 in the flowering time network, we used CRISPR/Cas9 genome editing to generate new alleles, which we termed ostrx1-2 and ostrx1-3. In these alleles, an adenine and thymine were inserted in exon 1 and exon 3, respectively (Fig. S2a,b), resulting in premature stop codons (Fig. S2b). As described in Choi et al. (2014), the ostrx1 mutants displayed a strong late-flowering phenotype in Hefei (LDs). The late-flowering phenotype existed in Lingshui (SD) and restricted SD conditions (Fig. S2c,d), suggesting that OsTrx1 might be involved in a photoperiod-independent pathway.

To determine the effects of these mutations, we first examined the transcript levels of the florigen genes RFT1 and Hd3a. We isolated total RNA from leaves of 80-d-old plants and subjected it to quantitative RT-PCR analysis. RFT1 and Hd3a transcript levels exhibited a strong diurnal rhythm in wild-type plants, whereas they were markedly reduced in ostrx1-2 and ostrx1-3 plants throughout the day (Fig. S2e).

RFT1 and Hd3a are regulated by Ehd1 and Hd1 (Yano et al., 2000; Doi et al., 2004; Ishikawa et al., 2005, 2011; Komiya et al., 2008, 2009), respectively. We therefore measured the mRNA levels of Ehd1 and Hd1, finding that the expression of Ehd1, but not Hd1, was abolished in ostrx1 plants. Ghd7, which acts as a negative regulator of Ehd1, was highly induced in ostrx1 plants. These results were consistent with the results of a previous study (Choi et al., 2014).

Next, we examined the expression of Ehd2, Ehd3, Ehd4 and EL1, finding that these genes were not affected in the ostrx1 mutants compared with the wild-type. The SD flowering activator OsMADS51, but not OsMADS50 or OsMADS56, was downregulated in ostrx1 mutants (Fig. S2e). We then examined the transcripts of Ehd1, RFT1, Hd3a, Hd1 and Ghd7 in SDs, and found that Ehd1, RFT1 and Ehd3a, but not Hd1 or Ghd7, were downregulated in ostrx1 mutants (Fig. S2f). These results suggest that OsTrx1 might be involved in regulating the heading date in rice through the Ehd1–RFT1 pathway under LD conditions.

OsTrx1 is required for H3K4me3 deposition at Ehd1

As OsTrx1 rescued the phenotype caused by the axl mutation, we investigated whether the reduction in transcript levels of the genes described above was caused by attenuated H3K4me3 in the ostrx1 mutants. We investigated the distribution of H3K4me3 by ChIP, followed by quantitative PCR measurement of DNA enrichment. Indeed, H3K4me3 levels were reduced at Ehd1, RFT1 and Hd3a in ostrx1 plants, indicating that OsTrx1 is required for H3K4me3 at these loci (Figs 1a,b, S3a,b). In contrast with Ehd1, RFT1 and Hd3a, the transcript levels of Ghd7 were induced in ostrx1 mutants (Choi et al., 2014) and it was found that the H3K4me3 level of Ghd7 was upregulated in ostrx1 plants (Fig. S3c,d).

To examine whether OsTrx1 binds to these loci, we conducted ChIP with OsTrx1. To this end, we generated a construct harboring the full-length OsTrx1 sequence fused to a FLAG tag driven by the native OsTrx1 promoter (ProOsTrx1:FLAG-OsTrx1). Transformation with this construct rescued the ostrx1 late-flowering phenotype, indicating that FLAG-OsTrx1 retains OsTrx1 function (Fig. S3e,f). We measured the occupancy of OsTrx1 via ChIP analysis with a specific anti-FLAG antibody, followed by quantitative PCR. OsTrx1 was enriched along the promoter and coding region of Ehd1, but not RFT1, Hd3a or Ghd7 (Figs 1c, S3g,h). These results indicate that OsTrx1 directly targets Ehd1 and increases the H3K4me3 levels of the Ehd1 locus.

To elucidate the relationship between OsTrx1 and Ehd1, we generated an ehd1 mutant using CRISPR/Cas9. In the ehd1-11 allele, an adenine was deleted in exon 3 of Ehd1, leading to an early stop codon in this mutant (Fig. S4a,b). The ehd1-11 plants exhibited a severe late-heading phenotype (Fig. 1d,e). We then examined the genetic relationship between OsTrx1 and Ehd1 by generating the ostrx1-2 ehd1-11 double mutant. The ostrx1-2 ehd1-11 mutant displayed a late-heading phenotype when grown in Hefei (LD) and Lingshui (SD), similar to the ehd1-11 single mutant (Fig. 1d,e), indicating that OsTrx1 is involved in an Ehd1-dependent pathway.

OsTrx1 interacts with SIP1 in vitro and in vivo

Given that OsTrx1 has no known DNA-binding domain, we therefore asked how OsTrx1 could target Ehd1. We investigated whether any transcription factors physically interact with OsTrx1 to allow it to target Ehd1. We fused OsTrx1 with a binding domain (BD) and screened its potential interactors using a cDNA library with the yeast two-hybrid system. Sequencing 10 positive colonies from screening revealed that two insertions were in the correct frame and able to encode proteins. One encoded a WD40-domain protein, and another encoded a protein containing a C2H2-type zinc finger domain (Fig. S5a), which we named SIP1 (SDG723/OsTrx1 Interaction Protein 1). SIP1 is encoded by LOC_Os09g38790. Phylogenetic analysis revealed that SIP1 is conserved in multiple plant species, including rice, Setaria italica, Brachypodium distachyon, Sorghum bicolor, maize (Zea mays) and A. thaliana, but not in mammalian cells (Fig. S5b).

We further confirmed the OsTrx1–SIP1 interaction by reciprocal yeast two-hybrid tests, BiFC and Co-IP experiments. First, we generated a vector harboring full-length SIP1 fused with AD, and found that OsTrx1 directly bound to SIP1 in yeast (Fig. 2a). For BiFC, functional YFP was observed in the nucleus in cells co-transformed with OsTrx1-YFP^N (fused with the N-terminal half of yellow fluorescent protein) and full-length SIP1-YFP^C (fused with the C-terminal half of YFP) (Fig. 2b). Immunoprecipitation using HA antibody revealed that OsTrx1 bound to SIP1 in rice protoplasts co-transformed with HA-OsTrx1 and FLAG-SIP1 (Fig. 2c).
We then investigated which domain of OsTrx1 is essential for the OsTrx1–SIP1 interaction. Yeast two-hybrid tests of different fragments of OsTrx1 showed that the C-terminus of OsTrx1, but not its N-terminus, bound to SIP1 (Fig. 2d,e). This yeast two-hybrid interaction was confirmed by a pull-down assay. Beads attached to SIP1 fused with His successfully bound to the C-terminus of OsTrx1 fused with GST, but not to the N-terminus of OsTrx1 fused with GST (Fig. 2f). In the complementary pull-down assay, the C-terminus of OsTrx1 fused with GST bound to His-fused SIP1 (Fig. 2g). These results indicate that OsTrx1 interacts with SIP1 in vitro and in vivo.

**Loss of SIP1 function leads to a late heading date**

To investigate the genetic function of SIP1 in regulating the heading date, we generated sip1 mutants using CRISPR/Cas9. Sequencing revealed that the sip1-1, sip1-2 and sip1-3 alleles have a cytosine insertion in exon 1, a thymine deletion in exon 1 and a guanine deletion in exon 2, respectively, which result in early stop codons (Fig. 3a,b). All three sip1 mutants showed a late-flowering phenotype when grown in Hefei (LD), Lingshui (SD) and restricted SD conditions (Fig. 3c,d), suggesting that SIP1 might also be involved in a photoperiod-independent pathway.

We then investigated the expression of genes in the flowering network in the sip1 mutants. RT-PCR results revealed that the transcript levels of Ehd1, RFT1, Hd3a and OsMADS51 were reduced, whereas the transcript level of Ghd7 was upregulated in the sip1 mutants compared with the wild-type. The transcripts of Hd1, Ehd2, Ehd3, Ehd4, OsMADS50, OsMADS56 and EL1 were not affected in the sip1 mutants (Fig. 3e). These observations are consistent with the late-flowering phenotype caused by sip1 mutation and suggest that SIP1 might be involved in the Ehd1–RFT1 pathway.

The possibility of a genetic relationship between OsTrx1 and SIP1 was investigated by crossing sip1-1 into ostrx1-2. The sip1-1 ostrx1-2 double mutant exhibited a late-flowering phenotype similar to that of ostrx1-2 (Fig. 4a,b). These results suggested that SIP1 and OsTrx1 might function together to regulate the same downstream genes. To further confirm these results, we examined the transcripts of Ehd1 in the sip1 ostrx1-2 double mutant. RT-PCR results revealed that the level of the Ehd1 transcript in sip1 ostrx1-2 was similar to
that in *ostrx1-2* (Fig. 4c). These results indicate that *SIP1* and *OsTrx1* act in the same pathway.

**SIP1 activates ProEhd1:GUS activity**

We next investigated whether SIP1 directly activates the expression of *Ehd1*, *RFT1* and *Hd3a* by assessing the activation of these genes by SIP1 using reporter constructs harboring *ProEhd1:GUS*, *ProRFT1:GUS* and *ProHd3a:GUS*. The vectors containing GUS driven by different promoters and 35S:LUC were co-transformed into rice protoplasts with or without SIP. *ProEhd1:GUS* activity, but not *ProRFT1:GUS* or *ProHd3a:GUS*, was highly induced in the presence of SIP1 (Fig. 5a,b). *OsTrx1* failed to activate *ProEhd1:GUS* activity, but *OsTrx1* did not reverse the SIP1-induced
Mutations in SIP1 result in a late heading date. (a) Gene structure of SIP1, indicating exons (boxes), introns (lines) and nucleotide insertions or deletions (triangles). (b) A nucleotide insertion in sip1-1 and a nucleotide deletion in sip1-2 and sip1-3 result in early stop codons in all three mutant alleles. The inserted and deleted nucleotides are marked in red, and stop codons caused by a shift in the open reading frame (ORF) are indicated in green. (c) Representative image of 103-d-old wild-type and sip1 mutants in long-day (LD) (Hefei). (d) Days to heading in wild-type and sip1 plants under LD (Hefei), short-day (SD) (Linshui) and restricted SD. Values shown are the mean ± standard deviation of heading days; 20 plants were scored per line. (e) Transcript levels of flowering network genes in sip1 mutants. RNA isolated from leaves of 80-d-old plants under restricted long-day conditions was used for RT-PCR. Closed bars, dark period; open bars, light period. ZT, Zeitgeber time. The y-axis shows the transcript level relative to rice Ubiquitin. Experiments were repeated at least three times, and the data from the representative experiment shown are presented as means ± SE, n = 3 replicates.
activation (Fig. 5a, c). These observations suggest that SIP1 might activate Ehd1 via its promoter.

**SIP1 binds to the Ehd1 promoter**

We therefore investigated whether SIP1 directly binds to the Ehd1 promoter. We fused SIP1 with an AD and used a yeast one-hybrid system to test whether it interacted with fragments of the Ehd1 promoter. SIP1 directly binds to the Ehd1 promoter region from −465 to −451, suggesting that this region is essential for SIP1 binding (Figs 5d, e, S6).

We then investigated the binding specificity of SIP1 via an EMSA. A retarded band was observed when SIP1 was present, whereas the mutated probes showed reduced binding (Fig. 5f, g). The shifted band was abolished when we used a specific competitor probe, but not mutated competitor probes (Fig. 5h), indicating that SIP1 binds to the promoter of Ehd1 in vitro. We investigated whether this motif could be found in the promoters of flowering time genes, but this motif was not present in the promoters of genes tested in sip1 plants.

**SIP1 is required for OsTrx1 occupancy at Ehd1**

To investigate whether SIP1 binds to Ehd1 in vivo, we generated an antibody specific to SIP1 (Fig. S7a) and assessed the binding of SIP1 to the Ehd1 promoter using ChIP analysis with the SIP1 antibody, followed by quantitative PCR analysis of DNA enrichment at multiple points along Ehd1. SIP1 was highly enriched at the Ehd1 promoter, but not in sip1 mutants (Fig. 6a). These results demonstrate that SIP1 can bind to Ehd1 in vivo.

As SIP1 binds directly to Ehd1 and activates its expression, we investigated whether SIP1 is required for OsTrx1 to target Ehd1, which, in turn, would affect H3K4me3 levels. We examined H3K4me3 levels by ChIP-PCR, finding that H3K4me3 of Ehd1 was reduced in sip1 (Fig. 6b), suggesting that SIP1 is required for the maintenance of proper H3K4me3 levels at Ehd1.

As OsTrx1 can accomplish H3K4me3 and interacts with SIP1, we then investigated whether SIP1 is required for the binding of OsTrx1 to Ehd1. We measured the enrichment of OsTrx1 via ChIP with a specific antibody (Fig. S7b, c), followed by quantitative PCR analysis of DNA enrichment. OsTrx1 enrichment was reduced in the sip1 mutants compared with the wild-type, indicating that the targeting of Ehd1 by OsTrx1 depends on SIP1 (Fig. 6c). We then investigated SIP1 enrichment and found that it was not affected in the ostrx1 mutant, suggesting that SIP1 binding to Ehd1 is independent of OsTrx1 (Fig. S7d).

**Discussion**

In this study, we have demonstrated that OsTrx1 interacts with the transcription factor SIP1 to modulate flowering, measured as the heading date, in rice. OsTrx1 interacts with SIP1, which allows OsTrx1 to increase H3K4me3 levels at Ehd1. Defects in OsTrx1 and SIP1 result in a late heading date under both LD and SD conditions, and these two factors are involved in the Ehd1–RFT1 pathway. These results were further supported by the finding that defects in SIP1 and OsTrx1 reduce the expression levels and H3K4me3 levels of Ehd1. Together, these results provide compelling evidence that SIP1 recruits OsTrx1 to modulate the heading date by regulating the expression level and H3K4me3 level at Ehd1.

In mammalian cells, MLL1 directly binds DNA via its CXXC domain. However, no known CXXC domain is present in yeast SET1 and the plant TRITHORAX family proteins ATX1, ATX2 and OsTrx1. ATX1 participates in pre-initiation complex formation at the promoter and adds the H3K4me3 modification in the gene body (Ding et al., 2011b, 2012b; Fromm & Avramova, 2014). ATX1 has multiple domains, including Tudor, DAST, PWWP, ePHD, Win and SET, which have diverse functions. The ePHD domain affects expression from specific ATX1-dependent genes through its ability to bind the lipid phosphatidyl inositol-5 phosphate (PtdIns5P), influencing the nuclear cytoplasmic distribution of ATX1 (Alvarez-Venegas et al., 2006;
Fig. 5 SIP1 binds to the Ehd1 promoter. (a) Vectors used in the GUS activity assay. (b) (l)-Glucuronidase (GUS) activity from the ProEhd1:GUS, ProRFT1:GUS and ProHd3a:GUS reporter constructs for cells transformed with SIP1. The x-axis shows the relative GUS activity compared with the internal luciferase control (35S:LUC). Asterisks indicate $P < 0.05$ by t-test. (c) GUS activity from the ProEhd1:GUS reporter constructs for cells transformed with SIP1 and OsTrx1. The x-axis shows the relative GUS activity compared with the internal luciferase control (35S:LUC). Asterisks indicate $P < 0.05$ by t-test. (d) Diagram of the Ehd1 promoter showing its different regions. The position of each region is marked on the left. (e) Yeast one-hybrid assay revealing that 15 base pairs in the Ehd1 promoter are essential for SIP1 binding. Yeast grown on 0 mM Aureobasidin A (AbA) was used as a negative control. (f) The sequences of probes used for electrophoretic mobility shift assay (EMSA). The conserved motif TCAAATTTTATTTGT is shown in blue, and the mutated nucleotides are shown in red. The motif is located in P3 of Fig. 2(a). (g, h) Gel shift assay using SIP1 and various probes. The ability of SIP1 to bind to different mutated probes labeled with $^{32}$P was assessed (g), and this binding specificity was tested by adding unlabeled wild-type competitor probe or mutated probes (h).
OsTrx1 rescues the developmental defects caused by the atx1 mutation, suggesting that OsTrx1 and ATX1 have similar functions.

Previous results have suggested that OsTrx1 promotes flowering via the repression of the expression level of Ghd7 (Choi et al., 2014). The PHD domain of OsTrx1 directly binds to H3 and the C4HC2H motif of OsTrx1 interacts with Ehd3 containing a PHD finger motif. Defects in OsTrx1 might disrupt Ehd3’s ability to repress Ghd7, resulting in late flowering (Choi et al., 2014). Given that the H3K4me3 level is correlated with transcriptional activation (Ding et al., 2011b; Fromm & Avramova, 2014), the induced transcription levels and reduced H3K4me3 levels of Ghd7 suggest that Ghd7 might not be the direct target of OsTrx1. A recent study has indicated that OsWDR5a interacts with the Win motif of OsTrx1 and directly binds to histone H3 (Jiang et al., 2018). Plants in which OsWDR5a expression is decreased by RNA interference produce fewer secondary branches and less grain, and exhibit a delayed heading date under LD and SD conditions, whereas loss of OsWDR5a function results in embryo lethality. OsWDR5a binds to Ehd1 to regulate its H3K4me3 and expression levels (Jiang et al., 2018), which is consistent with our current study.

Our study indicates that OsTrx1 interacts with the C2H2 zinc finger protein SIP1. SIP1 binds to the promoter of Ehd1 and recruits OsTrx1 to accomplish H3K4me3 at Ehd1. In Arabidopsis, transcriptional factor VAL1, interacting with LHP1 and binding to FLOWERING LOCUS C (FLC), results in the mediation of H3K27 trimethylation at FLC (Questa et al., 2016; Yuan et al., 2016). The transcription factor SUF4 interacts with MOS1 (MODIFIER OF sncl) to regulate the flowering time (Li et al., 2010; Bao et al., 2014). Recent results have supported the idea that transcriptional factors are critical for histone phosphorylation to regulate flowering time and hypocotyl elongation in Arabidopsis (Su et al., 2017; Zheng & Ding, 2018; Zheng et al., 2018).

In A. thaliana, a model LD plant, control of floral transition by endogenous (autonomous, gibberellin, circadian clock, age, sugar budget) and environmental (vernalization, ambient temperature, photoperiod) cues is a key factor in adaptation to different regions (Izawa, 2007; Bluemel et al., 2015). The floral repressor FLC and the floral promoter CONSTANS (CO) control the florigen gene FT; FLC and CO are key transcriptional regulators of the flowering pathways. Mutations in ATX1 lead to early flowering as a result of reduced transcript levels of FLC (Pien et al., 2008; Jiang et al., 2009).

Rice, a model SD plant, contains two major flowering pathways: a conserved photoperiod pathway and a unique photoperiod-independent time pathway. Ehd1 is an evolutionarily unique gene in rice that does not have an ortholog in Arabidopsis. The regulation of Ehd1 expression also involves a gene network distinct from that in Arabidopsis (Doi et al., 2004; Tsuji et al., 2011). Ehd1 promotes the expression of RFT1, and Ehd1 transcript levels increase in the presence of OsGI, Ehd2, Ehd3 and Ehd4 under LDs. By contrast, Ehd1 promotes the expression of H4S3a, and Ehd1 transcript levels increase in the presence of OsGI under SD conditions (Doi et al., 2004; Komiya et al., 2009; Tsuji et al., 2011). In the current study, OsTrx1 and
SIP1 were identified as new factors that positively regulate Ehd1, suggesting that the activation of Ehd1 requires factors involved not only in transcriptional regulation, but also in histone modification. Together, our findings indicate that SIP1 interacts with OsTrx1, which, in turn, regulates the expression of Ehd1 and heading date in rice.

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Author contributions

Y.D. and P.J. conceived the study and designed the experiments. P.J. performed most of the experiments. S.W. and H.Z. performed EMSA. H.Li performed the rice transformation. F.Z. performed the ChIP-PCR. Y.S. and Z.X. prepared the materials. P.J. performed most of the experiments. S.W. and H.Z. contributed equally to this work.

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Supporting Information

Additional Supporting Information may be found online in the Supporting Information tab for this article:

Fig. S1 Complementation of atxl-1 with OsTrxl1.

Fig. S2 Generation of ostrxl1 mutants using CRISPR/Cas9.
Fig. S3 H3K4me3 profiles at RFT1, Hd3a and Ghd7.

Fig. S4 Generation of the ehd1 mutant using CRISPR/Cas9.

Fig. S5 SIP1 encodes a C2H2 zinc finger protein.

Fig. S6 Identification of SIP1 binding sites in the Ehd1 promoter.

Fig. S7 The specificity of the antibodies for SIP1 and OsTrx1.

Table S1 Average daylight in 10-day intervals in 2017 at Hefei and Lingshui, China

Table S2 The constructs and primers used in this study

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