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CONSTANS-like 9 (COL9) delays the flowering time in *Oryza sativa* by repressing the *Ehd1* pathway

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ABSTRACT

Flowering or heading is one of most important agronomic traits in rice. It has been characterized that CONSTANS (CO) and CONSTANS-like (COL) proteins are critical flowering regulators in response to photoperiodic stress in plants. We have previously identified that the COL family member *OsCOL9* can positively enhance the rice blast resistance. In the present study, we aimed to explore the functional role of *OsCOL9* in modulating the photoperiodic flowering. Our data showed that overexpression of *OsCOL9* delayed the flowering time under both short-day (SD) and long-day (LD) conditions, leading to suppressed expressions of *EHd1*, *RFT* and *Hd3a* at the mRNA Level. *OsCOL9* expression exhibited two types of circadian patterns under different daylight conditions, and it could delay the heading date by suppressing the *Ehd1* photoperiodic flowering pathway. In contrast, the expressions of previously reported flowering regulators were not significantly changed in *OsCOL9* transgenic plants, indicating that *OsCOL9* functioned independently of other flowering pathways. In addition, *OsCOL9* served as a potential yield gene, and its deficiency reduced the grain number of main panicle in plants. Furthermore, yeast two-hybrid assay indicated that OsCOL9 physically interacted with Receptor for Activated C-kinase 1 (OsRACK1). Rhythmic pattern analysis suggested that *OsRACK1* responded to the change of daylight, which was regulated by the circadian clock. Taken together, our results revealed that *OsCOL9* could delay the flowering time in rice by repressing the *Ehd1* pathway.

1. Introduction

Flowering initial marks the plant growth from the vegetative stage into the reproductive stage, and this transition determines whether the plants are able to produce sufficient seeds to extend the life of the species [1]. Plants are sensitive to climatic and environmental changes, and such a characteristic ensures them integrate internal signals with circumstance fluctuation, resulting in preciously controlled flowering time [2]. The molecular regulatory mechanism of plant flowering has been broadly surveyed in Arabidopsis thaliana. Different daylight conditions regulate the transcript abundance of phytochrome gene *PhyB* [3], which further suppresses the expression of central photoperiodic flowering regulator CONSTANS (CO) in Arabidopsis [4]. CO protein directly induces the expression of FLOWERING LOCUS T (FT) at the mRNA level [5]. As a major florigenic protein, FT coordinates with SUPPRESSOR OF OVER-EXPRESSION OF CONSTANT 1 (SOC1) to promote flowering in leaf vasculature [6]. Expression of *CO* is modulated by the circadian clock that sets its rhythmic cycling to reach a peak at the end of the day under the long-day (LD) condition. The circadian clock-complex GIGANTEA (GI), FLAVIN-BINDING, KELCH REPEAT, F-BOX 1 (FKF1) and CYCLING DOF FACTOR (CDF) play major roles in regulating daily *CO* expression [7–9]. Although the *CO* expression at the mRNA level is high during the night, but its expression at the protein level is not increased since the CO interacts with an E3 ubiquitin ligase CONSTITUTIVE PHOTOMORPHOGENIC 1 (COP1), leading to CO degradation by proteasome system [10].

Compared with Arabidopsis, rice is the short-day (SD) plant, and its flowering time is jointly modulated through two different photoperiodic flowering pathways. *OsGI* [11] and heading date 1 (*Hd1*) [12] are orthologs of the Arabidopsis *GI* and *CO*, respectively. Three homologues of *FT* have been identified to promote flowering in rice genome, including *Hd3a*, RICE FLOWERING LOCUS T 1 (*RFT1*) and FT-like 1 (*FTL1*) [13]. The *OsGI-Hd1-Hd3a* regulatory pathway is similar to the *GI-CO-FT* model in Arabidopsis. *Hd1* promotes the

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Hd3a expression under the SD condition, whereas *CO* has opposite effects on *FT* expression and flowering under the SD condition. In addition, a distinct flowering pathway mediated by grain height date 7 (*Ghd7*)-early heading date 1 (*Ehd1*)-*RFT1* has been identified in rice. *Ghd7* independently suppresses the *Ehd1* expression and delays the heading date under the LD condition [14]. *Ehd1* not only directly promotes the *RFT1* expression, but also up-regulates the *Hd3a* expression under the LD condition in *Hd1*-deficient plant [15]. However, *OsGI* is an essential regulator in the photoperiodic flowering pathway induced by of *Ehd1* and *Hd3a* [16].

A total of 17 *COL* genes have been identified in rice genome by bioinformatics analysis [17,18]. COL family proteins play a key role in rice photoperiodic flowering. *Hd1* has a parallel function in the regulation of flowering time, but it represses the flowering under the LD rather than SD condition [19]. *OsCO3* primarily controls flowering time under the SD condition by negatively regulating the expressions of *Hd3a* and *FTL*, which is independent of the SD-promotion pathway [20]. *OsCOL4* is a constitutive repressor functioning upstream of *Ehd1*, and *OsphyB* dramatically decreases the *OsCOL4* expression [21]. *DTH2* encodes a COL protein and functions independently of the known floral integrators *Hd1* and *Ehd1*, and it can promote early heading and increase reproductive fitness under the natural LD condition [22].

We have previously found that *OsCOL9* positively modulates the rice blast resistance through SA and ETH pathways. *OsCOL9* encodes a COL protein consisting of one BBOX and one CCT domain. OsCOL9 is localized to the nucleus and has the transcription activation activity. However, we found that *OsCOL9* overexpression delays the heading date under SD and LD conditions. In the present study, we explored the molecular mechanism of *OsCOL9* in regulation of heading date in rice under SD and LD conditions.

2. Materials and methods

2.1. Generation of the OsCOL9-OX and oscol9-ko transgenic plants

The full-length of *OsCOL9* cDNA was isolated rice plants using the cDNA F/R primers encompassing translation start and stop codons. This cDNA insert was cloned between the maize ubiquitin promoter and *Nos* terminator of the plant expression vector pOX. *CRISPR/Cas9* technology was used to generate *OsCOL9* knock-out plants. As reported by Ma et al. pOX-OsCOL9 and pYLCRISPR/Cas9-OsCOL9 were then introduced into agrobacterium strain EHA105 and then transformed to wild-type (*Pik-H4* NILs) calli. Transgenic rice plants were regenerated from the transformed callus, and then the *OsCOL9* is overexpressed or knocked out in the obtained transgenic rice plants were further confirmed with target site sequencing and qRT-PCR.

2.2. Plant materials and growth condition

The oscol9-ko and OsCOL9-OX were previously obtained, and the wild-type was rice blast resistance line *Pik-H4* NILs. Wild-type and OsCOL9 transgenic plants were maintained in a growth chamber at 28 °C under the LD condition (14:10 h light: dark photoperiod) for 50 days. Subsequently, plants were grown under the SD condition (10:14 h light: dark photoperiod) until flowering. The heading date was recorded as the day when the panicle grew out from the node.

2.3. Real-time PCR analysis of gene expression

Total RNA was extracted from 100 mg of rice seedling with TRIZOL@Reagent (Invitrogen, Beijing, China), and purified RNA was reversely transcribed into cDNA using PrimeScript™ RT reagent Kit (Takara, Dalian, China) according to the manufacturer's instructions. Quantitative amplification was performed in a 20-μL reaction system containing 10 μL of SYBR Premix ExTaq™ (TaKaRa, Dalian, China) using an AB Stepone plus real-time PCR detection system. Relative gene expression was calculated using the $2^{-\Delta\Delta CT}$ method.

2.4. Investigation of the plant agronomic traits

The wild-type and *OsCOL9* transgenic plants reached the ripening stage at 90 days after seed germination under both SD and LD conditions. During the late stage of grain development, several agronomic traits were investigated, including plant height, panicle length, grain number per panicle, 1000-grain weight, and primary branch number of main panicle.

2.5. Yeast two-hybrid (Y2H) assay

OsCOL9 $BBOX_{1\text{-}150aa}$ and $CCT_{301\text{-}422aa}$ were respectively cloned into the BD plasmid pGBKT7 by homologous recombination in Y2HGold yeast strain. The interaction of proteins from rice Y2H c-DNA library was screened using the yeast cells containing the resulting constructs as bait according to the manufacturer's instructions of Clontech Y2H handbook. The transformed yeast cells were cultured on SD/-Trp/-Leu and SD/-Trp/-Leu/-His+3AT plates and examined after inoculation for 3 days.

3. Results

3.1. OsCOL9 negatively regulates heading date

We first investigated the flowering time of *OsCOL9* transgenic plants (Fig. S1) under SD and LD conditions. The average heading date of the wild-type plant was 62 days and 71 days after the seed germination under SD and LD conditions, respectively (Fig. 1A–D). Compared with the wild-type plant, oscol9-ko flowered 1 week earlier under both SD and LD conditions (Fig. 1A–D). However, OsCOL9-OX remained at the vegetative stage after the flowering of wild-type plant, especially under the LD condition (Fig. 1A–D). Based on above-mentioned findings, we concluded that the *OsCOL9* negatively regulated the heading date of rice.

Previously studies have proved that the heading date of rice is regulated by *Hd1-Hd3a* and *Ehd1-RFT* flowering pathways. Therefore, we examined the expressions of *Hd1*, *Hd3a*, *Ehd1* and *RFT* in wild-type and *OsCOL9* transgenic plants. In oscol9-ko, the expressions of *FT* orthologs (*Hd3a* and *RFT*) was significantly greater compared with the wild-type plant, but the expression of *EHd1* was diminished in OsCOL9-OX (Fig. 1E–G). Therefore, we hypothesized that *OsCOL9* probably repressed the *Ehd1* expression, and such an inhibition was not restricted by different daylight conditions.

3.2. OsCOL9 functions as an upstream repressor of Ehd1

To understand the circadian expression pattern of *OsCOL9* under SD and LD conditions, we extracted the total mRNA from the leaf blade of wild-type and *OsCOL9* transgenic plants and then examined the gene expression by quantitative real-time PCR at 4-h intervals. Under the SD condition, the *OsCOL9* expression maintained at a low level, but its expression in daytime was greater compared with night-time (Fig. 2A). In contrast, the *OsCOL9* expression peaked at 12 p.m., and it was gradually decreased until at 20 p.m. under the LD condition (Fig. 2B). The circadian expression pattern suggested the abnormality in *OsCOL9* transgenic plant.

We further investigated the circadian expression patterns of *Hd1*, *Hd3a*, *Ehd1* and *RFT* in OsCOL9 transgenic plants. The

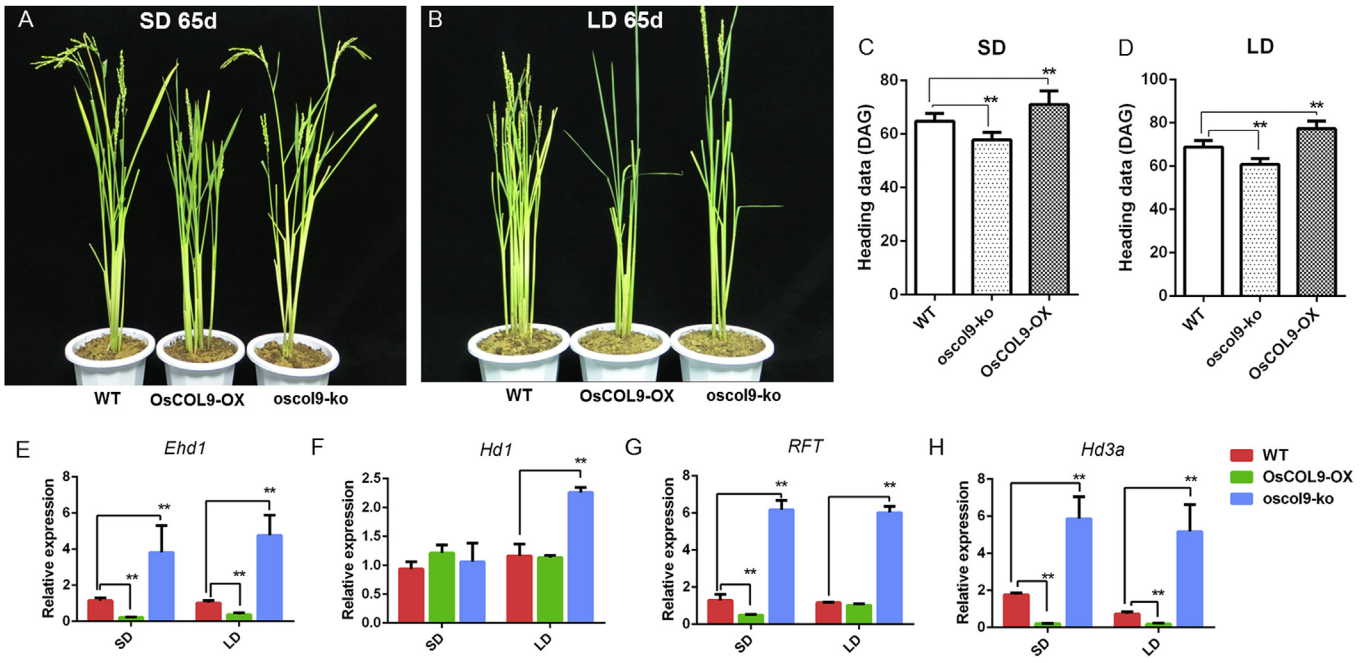

Fig. 1. *OsCOL9* delays the flowering time under both SD and LD. (A)–(B) Phenotypes of wild-type and *OsCOL9* transgenic lines at heading stage under different day length conditions. (C)–(D), Days to heading wild-type plants and *OsCOL9* transgenic lines under both SD and LD. Days to flowering was scored when first panicle bolted. Error bars indicate standard deviations, n = 15 plants. (E)–(H) Quantitative real-time PCR analyses of *Ehd1*, *Hd1*, *RFT*, and *Hd3a* transcripts level in wild-type and *OsCOL9* transgenic plants. Values shown are means ± SD, and asterisks indicate a significant difference according to the *t*-test (*P < 0.05; **P < 0.01) compared with wild-type.

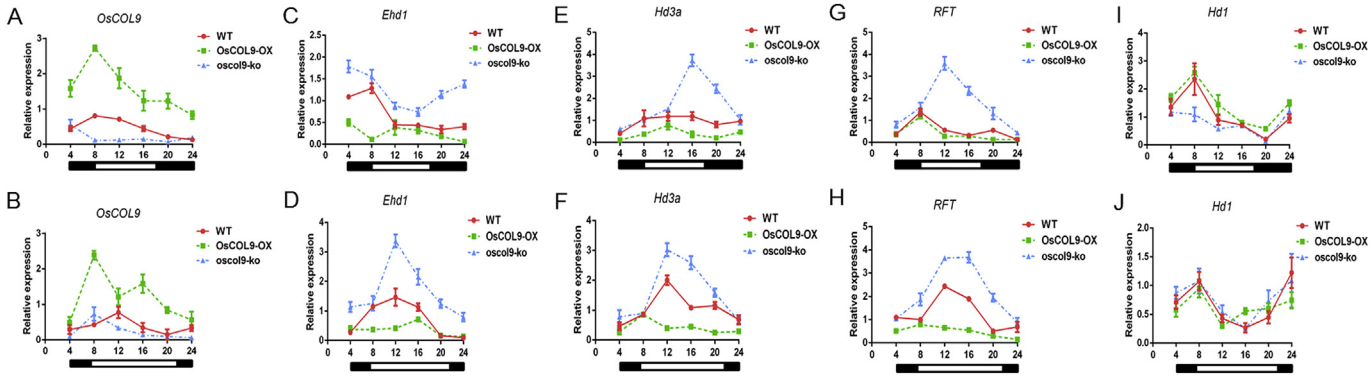

Fig. 2. Circadian expression pattern of *OsCOL9*, *Ehd1*, *Hd1*, *Hd3a*, and *RFT* in wild-type and *OsCOL9* transgenic plants under different day length conditions. (A)–(B) Circadian expression pattern of *OsCOL9* under SD and LD. (C)–(J) Relative expression of *Ehd1*,*Hd3a*,*RFT*, and *Hd1* in different plant materials under SD and LD. Leaf blades total RNA were extracted from wild-type, OsCOL9-OX, and oscol9-ko grown for 35 days and 45 days under SD and LD, respectively. Values shown are means ± SD of three independent experiments.

expressions of *Ehd1*, *Hd3a* and *RFT* were significantly increased in oscol9-ko under both SD and LD conditions (Fig. 2C–J). However, the expression of *Hd1* was barely changed in *OsCOL9* transgenic plants compared with wild-type plant under same daylight condition (Fig. 2C–J). These results demonstrated that *OsCOL9* functioned as an upstream repressor of Ehd1, and the decreased *Ehd1* expression delayed the flowering time.

We further investigated the expressions of *OsCOL9, Ehd1, RFT* and *Hd3a* in *OsCOL9* transgenic and wild-type plants at different development stages. The expression of *OsCOL9* and *Ehd1* was gradually increased and peaked at 6 weeks after germination under both SD and LD conditions, and then it was rapidly decreased at the end of the floral transition (Fig. S2). Associated with the phenotype of OsCOL9 transgenic plants, we concluded that OsCOL9 delayed the initial transition from the vegetative phase into reproductive stage by negatively regulating the *Ehd1* expression.

3.3. *OsCOL9 functions independently of other flowering regulators*

It is well known that multiple regulators are involved in the regulation of rice flowering [23]. Therefore, we assessed the expressions of many photoperiodic response factors that have been previously reported. Photochorme genes play important roles the regulation of flowering. *OsphyB* has been reported as an upstream repressor of *Hd1* [24]. We found that the expression of *OsPhyB* was not significantly changed in OsCOL9-OX and oscol9-ko compared with wild-type plant (Fig.S3G–H). Circadian clock genes control photoperiodic flowering response through clock regulation. We showed that the expressions of circadian clock genes (*OsTOC1*, and *OsPRR95*) were similar in *OsCOL9* transgenic and wild-type plants [25,26] (Fig.S3I–J and Fig. S4).

However, the relative expressions of early heading date family genes (*Ehd2* [27], *Ehd3* [28] and *Ehd4* [29]) and MADS-box family

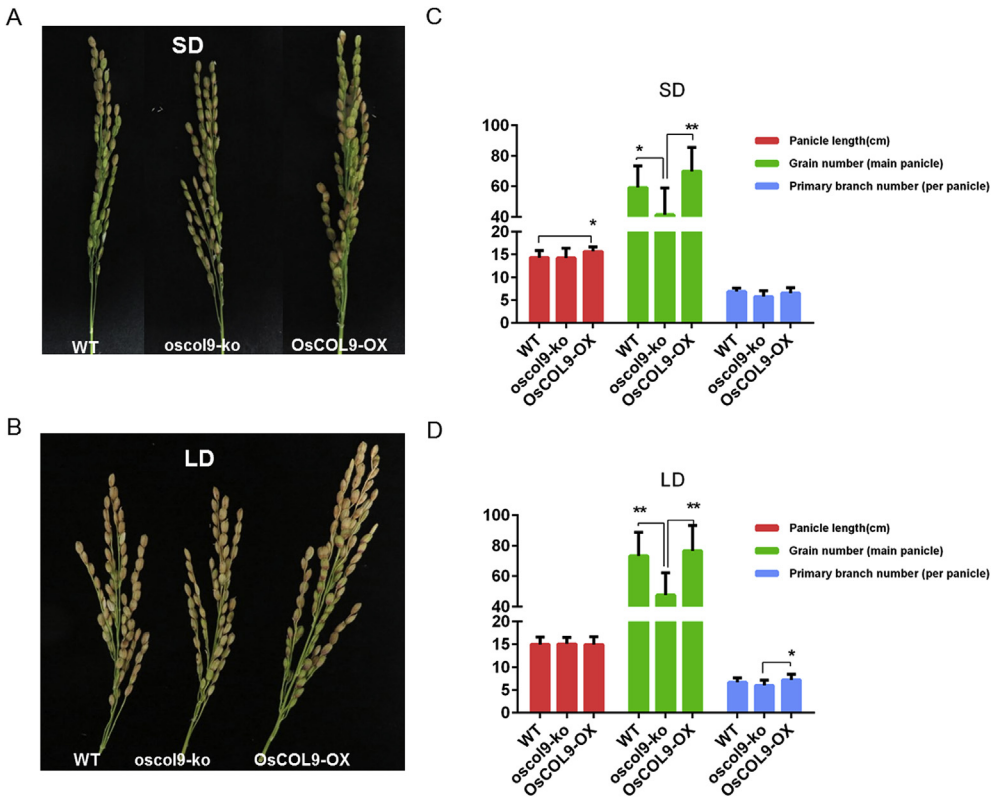

Fig. 3. *OsCOL9* improves the rice grain number of main panicle. (A)–(B) Main panicle phenotypes of wild-type, OsCOL9-OX, and oscol9-ko under both SD and LD. (C)–(D), Comparison of panicle length, grain number, and primary branch number between wild-type and *OsCOL9* transgenic lines under both SD and LD. Values are mean ± SD (n = 20). Asterisks indicate statistically significant differences compared with wild-type plants (*T*-test: *P < 0.05; **P < 0.01).

genes (*MADS14* and *MADS15*) [30]were weakly increased in *OsCOL9*-deficient plant under both SD and LD conditions (Fig. S3 A,B,E,F and Fig. S4). The expressions of other known floral genes still were maintained at a stable level, whereas the *DTH8* [31] expression was greater in OsCOL9-overexpressing plant (Fig.S3 C–D). Based on this finding, we believed that OSCOL9 functioned independently of most known flowering regulators, but it had subtle effects on the expressions of *DTH8*, *Ehd2* and *MADS14* (Fig. S3).

3.4. *OsCOL9 improves the rice grain number of per panicle*

COL genes play an important role in modulating the biomass of cereals by exerting an effect on their basic vegetative growth, thereby yielding grain [32]. We investigated the agronomic traits of *OsCOL9* transgenic and wild-type plants under both SD and LD conditions. Our results indicated that deficiency of *OsCOL9* negatively regulated the plant height, grain number of per panicle and 1000-grain weight, and obviously affected the grain size under the SD condition (Fig. S5). However, the primary branch number, and main panicle length were not affected in *OsCOL9* deficient plant under both SD and LD conditions (Fig. 3C, D). In contrast, the grain number of per panicle and 1000-grain weight in *OsCOL9*-overexpressing plant were significantly increased compared with *OsCOL9*-deficient plant under different photoperiod conditions (Fig. 3A–D). According to these data, we concluded that *OsCOL9* positively increased the rice grain yield through exerting an effect on grain number, and such an effect was not restricted by different photoperiod conditions.

3.5. *Identification of OsCOL9 interacting proteins by Y2H assay*

To illustrate the underlying molecular mechanism of OsCOL9-mediated flowering time, we performed the Y2H assay using *OsCOL9* $BBOX_{1\text{-}150aa}$ or $CCT_{301\text{-}422aa}$ ($OsCOL9_{151\text{-}300aa}$ holds the transcription activity) as bait to screen the interacting proteins of OsCOL9 from rice AD-cDNA library. A total of eight positive clones were obtained, including two transposon proteins, three WD40-containing proteins, one auxin-repressed family protein, one mitosis protein and one polypyrimidine tract-binding protein (Table S1). However, only the *OsRACK1* (LOC_Os01g49290) has been reported, and its encoding protein contains seven tandem WD40 repeats and physically interacts with Rac1 to enhance rice blast resistance [33].

In addition, we investigated the circadian expression pattern of *OsRACK1* in wild-type and OsCOL9 transgenic plants under both SD

Fig. 4. OsCOL9 interacts with OsRACK1 and delays the rice flowering time through repressing the *Ehd1* pathway under both SD and LD conditions.

and LD conditions. In wild-type plant, the *OsRACK1* expression was increased from the beginning of dark and peaked at 8 p.m., and then its expression was gradually decreased until the end of daylight (Fig. S6 A, B). However, the expression of *OsRACK1* remained unaffected in *OsCOL9* transgenic plants, and the changing trend was similar under both SD and LD conditions. This finding indicated that the *OsRACK1* expression was regulated by the circadian clock.

4. Discussion

4.1. OsCOL9 delays flowering time as an upstream repressor of Ehd1

In the present study, we found that *OsCOL9*-deficient plant exhibited an early-flowering phenotype under two different photoperiod conditions, whereas the *OsCOL9*-overexpressing plant displayed late-flowering phenotype. This finding on *OsCOL9* was similar to other *OsCOL* genes [20]. *OsCOL4* is a constitutive repressor functioning upstream of *Ehd1* under SD and LD conditions [21]. *DTH2* functions independently of the floral integrators *Hd1* and *Ehd1* [22]. However, *OsCOL9* deficiency significantly induced the *Ehd1* expression, these results indicated that *OsCOL9* mainly acted as an upstream repressor of *EHd1*, thereby leading to the up-regulation of *Hd3a* and *RFT* in *OsCOL9*-deficient plant (Figs. 2 and 4).

4.2. OsCOL9 positively regulates grain number

Rice heading date and yield are two distinct traits regulated by different quantitative trait loci (QTLs) [34]. However, evidence suggested that many regulators simultaneously mediate flowering time and yield. *Ghd7*, a COL family member, is a LD-specific repressor and can improve rice yield by accumulating second spike branch [32]. Our results suggested that overexpression of *OsCOL9* obviously improved the grain number of per main panicle, and *OsCOL9* was a positive regulator of rice yield (Fig. 3). With further analysis using Y2H, we found that OsCOL9 interacted with a putative uncharacterized protein (LOC_Os11g44810) which has been predicted as an auxin repressed protein (ARP1), and its ortholog *EBE* dramatically affects shoot architecture in Arabidopsis [35]. We supposed that OsCOL9 promoted the vegetative growth, and the increased yield could be attributed to ARP1-associated auxin signaling pathway (Table S1).

4.3. OsCOL9 interacts with WD40-containing protein OsRACK1

WD40 domain is highly conserved in eukaryotic organisms, which usually contains several tandem repeated sub-units with functional diversity in multiple pathways [36]. OsRACK1 contains seven tandem WD40 repeats, which interacts with OsCOL9 and probably recruits E3 ligase complex to mediate its degradation through the ubiquitin proteasome system. Moreover, clear evidence shows that WD40-containing protein RACK1 induces the RANKL-dependent activation of p38 MAPK ubiquitin pathway, which has sufficiently proved our assumption [37,38].

In summary, we characterized COL gene *OsCOL9*, which was involved in manipulation of rice flowering time. Our data suggested that *OsCOL9* delayed the rice heading through repressing the *Ehd1* pathway under both SD and LD conditions (Fig. 4), and it could positively regulate the grain number. In addition, OsRACK1 interacted with OsCOL9 in response to circadian photoperiod (Fig. 4), which probably mediated the OsCOL9 degradation and played an important role in regulation of rice flowering.

Conflict of interest

The authors declare that there is no conflict of interest.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2016.09.013.

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