CURVED CHIMERIC PALEA 1 encoding an EMF1-like protein maintains epigenetic repression of OsMADS58 in rice palea development

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SUMMARY
Floral organ specification is controlled by various MADS-box genes in both dicots and monocots, whose expression is often subjected to both genetic and epigenetic regulation in Arabidopsis thaliana. However, little information is known about the role of epigenetic modification of MADS-box genes during rice flower development. Here, we report the characterization of a rice gene, CURVED CHIMERIC PALEA 1 (CCP1) that functions in palea development. Mutation in CCP1 resulted in abnormal palea with ectopic stigmatic tissues and other pleiotropic phenotypes. We found that OsMADS58, a C-class gene responsible for carpel morphogenesis, was ectopically expressed in the ccp1 palea, indicating that the ccp1 palea was misspecified and partially acquired carpel-like identity. Constitutive expression of OsMADS58 in the wild-type rice plants caused morphological abnormality of palea similar to that of ccp1, whereas OsMADS58 knockdown by RNAi in ccp1 could rescue the abnormal phenotype of mutant palea, suggesting that the repression of OsMADS58 expression by CCP1 is critical for palea development. Map-based cloning revealed that CCP1 encodes a putative plant-specific EMBRYONIC FLOWER1 (EMF1)-like protein. Chromatin immunoprecipitation assay showed that the level of the H3K27me3 at the OsMADS58 locus was greatly reduced in ccp1 compared with that in the wild-type. Taken together, our results show that CCP1 plays an important role in palea development through maintaining H3K27me3-mediated epigenetic silence of the carpel identity-specifying gene OsMADS58, shedding light on the epigenetic mechanism in floral organ development.

Keywords: rice, MADS-box, H3K27 trimethylation, epigenetic regulation, EMF1-like, floral organ identity.

INTRODUCTION
Indicot plants, flowers generally consist of four whorls of organs from outside to inside: sepals, petals, stamens, and carpels. During the past two decades, extensive genetic and molecular studies have established an ABCDE model to explain the molecular mechanism underlying flower development in several eudicot species (Coen and Meyerowitz, 1991; Weigel and Meyerowitz, 1994; Theissen and Saedler, 2001; Solitis et al., 2007; Litt and Kramer, 2010). The temporal and spatial regulations of A-, B-, C-, D- and E-class genes and their combinations generate the proper floral pattern by specifying the identity of each floral organ. In Arabidopsis, for example, the A-class genes APETALA1 (AP1) and AP2 specify sepal identity, the B-class gene AP3 and PISTILLATA (PI) act together to specify petal identity and directly regulate AP1, while the combinative functions of B- and C-class genes specifies stamen identity (Mandel et al., 1992; Gustafson-Brown et al., 1994; Jofuku et al., 1994; Sundstrom et al., 2006). In addition to regulate the identities of stamen and carpel, the C-class gene AGAMOUS (AG) affects floral meristem determinacy and interacts with the B
and C function factors (Bowman et al., 1989, 1991; Drews et al., 1991; ÖMæoliéidigh et al., 2013). The D-class gene SEEDSTICK (STK), whose expression is exclusively limited to ovules, controls ovule identity redundantly with other two MADS-box genes SHATTERPROOF1 (SHP1) and SHP2 (Losa et al., 2010). The E-class genes SEPALLATA1/2/3/4 (SEPI/2/3/4) plays redundant role in determining the identity of all four whorls of floral organs and in regulating floral meristem determinacy (Pelaz et al., 2000; Ditta et al., 2004).

As a grass species, rice (Oryza sativa L.) has different floral architecture from dicots, as its spikelets are the structural units of the inflorescence. A typical rice spikelet consists of a pair of rudimentary glumes, a pair of empty glumes and a floret that is comprised of one palea, one lemma, two lodicules, six stamens and one pistil (Bommert et al., 2005; Itoh et al., 2005). Nowadays more and more floral homeotic genes have been identified in rice, and evidences from these genes suggest that the ABCDE model is partially applicable in rice. Nevertheless, the homology between the lemma/palea to sepal still remains controversial (Schmidt and Ambrose, 1998; Kyozuka et al., 2000; Ferrario et al., 2004; Luo et al., 2005; Thompson and Hake, 2009; Yun et al., 2013). The lemma and palea are grass-specific perianth organs with similar outer morphology but different size and number of vascular bundles. Besides, the lateral margins of palea (marginal region of palea, mrp) are membranous without trichomes, which are distinct from the central region of palea (Prasad et al., 2001). Therefore, it has been proposed that both common and distinct factors determine the identity of palea and lemma (Jeon et al., 2000; Ohmori et al., 2009; Sang et al., 2012). For example, OsMADS15/DEGENERATIVE PALEA (DEP), an AP1-like gene, specifies palea and sterile lemma identity (Wang et al., 2010) and a SEP-like gene OsMADS1/LEAFY HULL STERILE (LHS1) controls both cell proliferation and differentiation during lemma and palea development and floret meristem specification (Jeon et al., 2000; Prasad et al., 2005; Khanday et al., 2013). Phylogenetic analyses have demonstrated that two rice D-class MADS genes OsMADS13 and OsMADS21 cluster with Arabidopsis STK in a same lineage, while only OsMADS13 was found to specify ovule identity (Dreni et al., 2007; Li et al., 2011a). Interestingly, it was reported that rice genome encodes two duplicated and partially sub-function-alized C-class genes, OsMADS3 and OsMADS58, as AG homologs. OsMADS3 plays predominant roles in stamen and ovule specification, while OsMADS58 maintains floral meristem determinacy and might regulates carpel identity redundantly with OsMADS3 and both of them genetically interact with OsMADS6 and OsMADS16 (Yamaguchi et al., 2006; Dreni et al., 2011; Li et al., 2011a; Yun et al., 2013).

Epigenetic regulation can affect genome functions via modifications of DNA and chromatin proteins without altering DNA sequences. Histone modifications, as one of these mechanisms, play important roles in multiple biological processes in plants (Pfluger and Wagner, 2007; Wu et al., 2009; Liu et al., 2010; Deal and Henikoff, 2011; Feng and Jacobsen, 2011), such as flowering transition (Kim et al., 2005; Zhao et al., 2005; Xu et al., 2008; Berre et al., 2009; Sun et al., 2012; Sui et al., 2013), floral organ development (Ding et al., 2007; Grini et al., 2009), shoot and root branching (Dong et al., 2008; Cazzonelli et al., 2009) and hormone response (Sui et al., 2012). Moreover, Polycomb group (PcG) protein-mediated epigenetic silencing is a general epigenetic regulatory mechanism conserved in plants and animals (Schwartz and Pirrotta, 2007; Kohler and Villar, 2008). In Arabidopsis, two Polycomb repressive complex 1 (PRC1) and PRC2, were identified and extensively studied. It has been known that PRC2 contains a histone methyltransferase responsible for methylaing histone H3 at lysine 27 (H3K27me3), while PRC1 binds to H3K27me3 and establishes the monoubiquitination of H2A (Ca et al., 2002; Muller et al., 2002; Schubert et al., 2005; Turck et al., 2007; Schlatowski et al., 2008). In addition to their conserved counterparts in animals, plant PcG complexes likely have or are associated with plant-specific components. For example, EMBRYONIC FLOWER 1 (EMF1), a plant-specific protein, participates in H3K27me3-mediated silencing of target genes by acting downstream of EMF2 and likely interacts with both the PRC1 RING-finger proteins and the PRC2 component MULTICOPY SUPRESSOR OF IRA1 (MSI1) (Calonje et al., 2008). One target, AG, is bind directly by EMF1 and both the promoters and intragenic regions of AG are required for its epigenetic repression in vegetative tissues (Calonje et al., 2008). Functions in genome-wide analysis indicated that the EMF1 binding pattern is similar to that of H3K27me3 modification and it effectively silences the genetic pathways necessary for growth and development and stress response by repressing key regulatory genes (Kim et al., 2010, 2012). Although the roles of PcG proteins in developmental processes in Arabidopsis have been extensively explored, whether homeotic genes that specify floral organ identity are epigenetically regulated during rice flower development remains elusive.

Here, we report that an EMF1-like gene CURVED CHIME- RIC PALEA 1 (CCP1), maintains palea development in rice. We showed that CCP1 represses carpel identity-specifying OsMADS58 in palea through maintaining H3K27me3-mediated epigenetic silencing. Our results reveal an epigenetic mechanism by which a putative PcG component represses the expression of floral homeotic gene to participate in flower development in the monocot model plant.

RESULTS

Phenotypic characterization of the ccp1 mutant

To understand the regulatory mechanisms underlying rice seed development, we isolated a mutant with abnormal floral organs from a γ-ray-induced mutation pool of Zheij
ing27 (japonica) (Yang et al., 2011). Compared with the wild-type, the ccp1 mutant showed decreased plant height but increased tillers (Figures 1a and S1), the panicle length, primary branch number and seed setting rate were all significantly decreased in ccp1 (Figures 1b and S1). The ccp1 spikelets/grains displayed abnormal morphology, and could be classified into three types: (i) curved palea, wrinkled lemma without seeds, (ii) curved palea with seeds, and (iii) short grains with open hulls (Figure 1c). These phenotypes of ccp1 could be affected by environmental factors such as temperature and photoperiod because the ratio of these phenotypic classes varied by regions and crop seasons in our experimental stations (Table S1).

To investigate the cellular morphology of ccp1 grain hull, we examined the cross-sections of the ccp1 and wild-type lemma/palea (Figure 1d). No obvious differences in the number of vascular bundle and cell morphology of the lemma were observed between ccp1 and the wild-type, except that the mrp was defective in ccp1, resulting in the lack of interlocking between the lemma and palea (Figure 1d). Besides, the seedling leaves and flag leaf angles of ccp1 were smaller than the wild-type plants (Figure S2a,b). It was reported that the erect leaf was a typical brassinosteroid (BR)-related phenotype in rice (Yamamuro et al., 2000; Hong et al., 2003), prompting us to evaluate BR response and examine the expression of BR-related genes (Jiang et al., 2012). We indeed found that ccp1 showed less response to exogenous BR treatment in lamina joint test (Figure S2c) and the rice BR receptor gene BRASSINOSTEROID-INSENSITIVE1 (OsBRI1) was down-regulated in ccp1 (Figure S2d), in comparison with the wild-type control. These data together implied the possible involvement of CCP1 in floral developmental and BR signaling pathways.

**CCP1 functions in maintaining palea identity**

To investigate spikelet development in ccp1 in detail, we used scanning electron microscopy (SEM) to examine spikelets in the early developmental stages of rice flowers as defined (Ikeda et al., 2004). Compared with the wild-type plants (Figure 2a–c), the ccp1 florets showed no obvious defect in earlier stages Sp5 and Sp6 (Figure 2d,e); whereas in stage Sp7 the ccp1 palea was shorter and poorly developed with the notable tendency of curving inward (Figure 2f). At heading stage, the curved palea of ccp1 exhibited broad green marginal region with stigma-like tissue on the edge (Figure 2g,h), instead of the transparent mrp in the wild-type palea (Figure 2i). The ccp1 palea developed more trichomes on the outer epidermis, especially on the joint of palea body and mrp (Figure 2g,h) than the wild-type (Figure 2i). Moreover, the epidermal cells of the ccp1 mrp (Figure 2i) was distinct from that of the wild-type mrp (Figure 2j), but intriguingly resembled that of the wild-type carpel (Figure 2k). The cells of the stigmatic tissue on ccp1 palea (Figure 2m) showed the similar morphology to that of the wild-type stigma (Figure 2n). These cell biological observations suggest that mutation in the CCP1 gene induced carpel-like identity in the palea.

**Molecular cloning of CCP1**

The ccp1 mutant was crossed with the wild-type (Zhejing27) and all F1 hybrids displayed the wild-type phenotype. In the F2 population, the segregation ratio of

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**Figure 1.** Characterization of the ccp1 mutant. (a) Adult plants (30 days after heading) of the wild-type (Zhejing27, WT) and ccp1. Bar = 10 cm. (b) Mature panicles (30 days after heading) of the wild-type and ccp1 plants. Bar = 1 cm. (c) Grains of the wild-type and ccp1. Three types of ccp1 grains were shown (from left to right: type 1, type II and type III). Note that type 1 ccp1 grain was empty without seeds. Bar = 1 mm. (d) Transverse sections of spikelet hulls of the wild-type and ccp1. Three types of sections were shown (from left to right: type 1, type II and type III). Note that type 1 ccp1 grain was empty without seeds. Bar = 100 μm.
wild-type to ccp1 plants showed typical Mendelian segregation (3:1), which indicated that the mutant phenotype was controlled by a single recessive gene. To identify the CCP1 gene, we used an F2 mapping population generated from a cross between ccp1 and Zhenshan97 (indica) to narrow the CCP1 locus to a 160-kb region between two mark-
ers M7-1 and M7-4 on chromosome 1 (Figure 3a). By sequencing the genomic DNA sequences of all predicted genes within this region, we identified a 2-bp deletion in the third exon of Os01g12890 (Figure 3b), creating a new site for PscI restriction enzyme that can generate different digestion patterns of the wild-type and ccp1 genomic DNA fragments (Figure 3c). This 2-bp deletion in ccp1 potentially resulted in a truncated protein due to the premature translational stop. We performed genetic complementation assay and confirmed that Os01g12890 is the CCP1 gene (Figure 3d).

The CCP1 gene is predicted to encode an EMF1-like protein, which shares 37% amino acid sequence similarity and 20% identity with that of Arabidopsis EMF1 (Figure S3). No significant homology was found with other known proteins in eukaryotes, indicating that CCP1, like EMF1, is plant-specific (Aubert et al., 2001).

Expression pattern of CCP1 and protein localization

To determine the expression pattern of CCP1, we employed quantitative RT-PCR (qRT-PCR) and GUS reporter assay to examine the CCP1 expression in the wild-type plants. Real-time qPCR analysis showed that CCP1 transcripts accumulated in all examined tissues, including roots, seedlings, stems, leaves and panicles at various developmental stages (Figure 4a). Consistent with these results, examination of expression pattern of CCP1::GUS fusion reporter in transgenic plants found that GUS staining signals were detectable from the above mentioned rice tissues, suggesting that CCP1 is ubiquitously expressed (Figure 4b). We next examined the subcellular localization of CCP1 protein. A construct harboring CCP1-GFP fusion was introduced into onion and tobacco epidermal cells by particle bombardment and Agrobacterium-mediated infiltration, respectively. The fluorescent signals of CCP1-GFP fusion protein were observed exclusively in the nucleus of onion and tobacco cells with a speckle-like pattern (Figure 4c), indicating that CCP1 predominantly is a nuclear protein.

**CCP1 negatively regulates OsMADS58 expression**

As the ccp1 palea appears to partially obtain carpel-like identity, we examined the expression of C-class genes OsMADS58 and OsMADS3 as well as DROOPING LEAF (DL) in the palea of ccp1 and wild-type plants, which are reported to be involved in carpel specification (Yamaguchi et al., 2004, 2006; Dreni et al., 2011; Li et al., 2011a,b). qRT-PCR analyses revealed that all of these genes were up-regulated in varying degrees while only OsMADS58 expression was extremely elevated in the ccp1 palea in comparison to that in the wild-type (Figure 5). To deter-
mine whether the altered expression of OsMADS58 is specific to palea, we checked its expression in 2-week-old seedlings and found it also went up greatly in the ccp1 seedlings (Figure 5). These results indicated that expression of OsMADS58 could be repressed by CCP1 both in early vegetative development and reproductive organs. Similarly, ectopic expression of AG in Arabidopsis also resulted in abnormal first-whorl organs with stigmatic papillae at the tips and ovules on the margins (Mizukami and Ma, 1992). It has been recognized that the DL gene is another important player in regulating carpel specification and its ectopic expression in carpel-like organs has been reported in several rice mutants (Nagasawa et al., 2003; Yamaguchi et al., 2004; Ohmori et al., 2009; Sang et al., 2012). However, the ectopic expression of DL in palea did not induce the formation of carpelloid tissues in palea in the previous studies (Sang et al., 2012). Interestingly, we also found that DL transcripts were ectopic accumulated in the ccp1 palea but hardly detected in the wild-type palea (Figure 5), which might indicate that the ccp1 palea has carpel identity. In addition, although the transcript level of OsMADS3 rised slightly, this may not be able to induce carpel formation in the palea as the ectopic expression of OsMADS3 did not

Figure 4. Expression pattern of CCP1 and subcellular localization of the CCP1-GFP fusion protein.
(a) Expression pattern of the CCP1 gene detected by real-time PCR. The experiment was repeated three biological times with similar results. Error bars represent the standard deviation (SD) (n = 3).
(b) GUS activity was detected in different tissues of the transgenic CCP1::GUS reporter plants.
(c) Subcellular localization of the CCP1-GFP fusion protein in onion (upper) and tobacco (lower) epidermal cells, indicating its nucleus localization. Error bars represent the SD (n = 3).

Figure 5. Comparison of transcript levels of OsMADS58, DL and OsMADS3 in the wild-type and ccp1. Transcript levels of OsMADS58, DL and OsMADS3 in the palea of young spikelets and OsMADS58 in 3-week-old seedlings were detected by real-time RT-PCR, with levels in the wild-type normalized as 1. The experiment was repeated three biological times with similar results. Error bars represent the SD (n = 3). Corresponding semi-quantitative RT-PCR results were shown in lower panels. ACTIN were used as controls. The same sample was used for analysis of OsMADS58, DL, OsMADS3 and ACTIN expression in the palea of the wild-type, as well as ccp1.

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cause any morphological alterations in the palea (Kyozuka and Shimamoto, 2002). Taken together, we speculated that the acquisition of carpel identity in the ccp1 palea is most likely associated with the ectopic expression of Os-MADS58.

**Misexpression of OsMADS58 is responsible for carpeloid palea development in ccp1**

As OsMADS58 was constitutively up-regulated in both vegetative tissues and floral organs in ccp1 and it has been shown that OsMADS58 plays a critical role in controlling carpel identity (Yamaguchi et al., 2006; Dreni et al., 2011), we hypothesized that the altered expression of OsMADS58 in ccp1 might be responsible for its defective floral organ development. To test this possibility, we generated transgenic rice plants that constitutively expressed OsMADS58 driven by the 35S promoter (OsMADS58-OE) (Figure S4a). Likewise, the overexpression of OsMADS58 resulted in reduced plant height (Figure 6a). Consistent with the previous observations, we found that the lemma/palea of the OsMADS58-OE plants was shorter than that of the wild-type plants and their palea was curved inward like ccp1 (Figure 6b). The broad green marginal region with stigmatic-like organ (carpel-like organ) was also observed in the OsMADS58-OE palea (Figure 6c). In addition, OsMADS58-OE plants developed much more trichomes at the junction of mrp and palea, compared with the wild-type (Figure 6d,e). We also detected the expression of DL in the OsMADS58-OE palea and found that the overexpression of OsMADS58 subsequently led to the up-regulation of DL, which may be either directly or indirectly (Figure S4c).

To further confirm that the enhanced expression of OsMADS58 in ccp1 caused the abnormal palea development, we introduced an RNAi construct targeting OsMADS58 (OsMADS58-RNAi) into the ccp1 mutant plant (Figure S4b). We found that these OsMADS58-RNAi/ccp1 plants, which showed down-regulated expression level of OsMADS58 near to the wild-type, developed normal spikelets and palea, similar to the wild-type (Figure 6f-h). Taken together, these results strongly suggest that the ectopic expression of OsMADS58 results in the abnormality of palea in ccp1.

**H3K27 trimethylation-mediated repression of OsMADS58 is dependent on CCP1**

Studies have shown that EMF1 is a plant-specific PRC1 component that is required for establishing or maintaining H3K27me3 at, if not all, some target genes of PRC2 in Arabidopsis (Calonje et al., 2008; Kim et al., 2010, 2012). To determine whether CCP1 is required for H3K27me3 in rice, we first measured the total H3K27me3 level in ccp1. Western blotting analysis showed that the global H3K27me3 level was markedly reduced in ccp1, compared with the wild-type (Figure 7a). Since H3K27me3-mediated AG repression is largely dependent on the presence of EMF1 during the vegetative development (Calonje et al., 2008) and OsMADS58 was overexpressed in ccp1, we then investigated whether OsMADS58 expression in rice seedling development is regulated by H3K27me3. Chromatin isolated from fresh tissue of wild-type and ccp1 plants were further immunoprecipitated with an anti-trimethyl histone H3K27 antibody followed by qRT-PCR detection using primers specific to different OsMADS58 genomic regions (Figure 7b). We found that the H3K27me3 level at the OsMADS58 locus was dramatically reduced in the ccp1 plants (Figures 7b and S5). As H3K27me3 has been generally considered to be associated with gene silencing, our results suggest that the H3K27me3-mediated repression of OsMADS58 is dependent on CCP1 (Figure 7c).

Furthermore, to explore the possibility of relation between CCP1 and putative PRC in rice, we conducted a yeast two-hybrid assay and found that CCP1 interacts with Os04g0450400 and Os03g0640100, two rice homologous proteins of AtRING1A/1B and AtMSI1 (Figure S6). However, the interaction and biological significance need to be confirmed and elaborated by more in vivo and in vitro experiments.

**DISCUSSION**

In this study, we identified a rice mutant ccp1 which displays aberrant florets with carpel-palea or pistil-palea mosaic organs; its curved palea acquires carpel-like identity, as determined by cell morphology and gene expression analysis. The CCP1 gene encodes an EMF1-like protein that regulates expression of the MADS-box gene OsMADS58 through H3K27me3 modification, which controls palea identity during rice floral organogenesis. Several important MADS-box genes have been identified to function in palea morphogenesis in rice. For example, mutation of OsMADS1/LHS1 could lead to the loss of mrp (Prasad et al., 2005) and the osmads32/cfo1 mutants displayed bent paleas with broad marginal regions (Sang et al., 2012). Our study therefore adds to the list of the genes affecting floral organ identity and development, and more importantly sheds light on epigenetic regulation of floral organogenesis genes in the model crop.

The mutation in CCP1 also affects other important agronomic traits such as plant height, tiller number, panicle length, primary branch number, seed setting and grain filling (Figure S1). These pleiotropic effects are consistent with the ubiquitous expression pattern of CCP1. Due to the indistinctive rescue of the dwarf phenotype in OsMADS58-RNAi/ccp1 plants (Figure 5f), we deduced that there should be other downstream factor(s) regulated by CCP1 that functions in other developmental processes. In support of this hypothesis, we observed the weakened BR response and down-regulation of OsBRI1 in the ccp1 mutant (Figure S2), suggesting that the BR signaling pathway...
might also be disturbed by the loss of CCP1 function, a possible explanation of the dwarfism of ccp1 plants. Further investigation on CCP1-mediated gene expression and suppressor screening of ccp1 will identify additional target genes that play roles in these developmental pathways.

Most MIKC-type MADS-box genes function in different flower developmental processes, such as meristem differentiation, floral organ specification, cell proliferation and elongation (Ng and Yanofsky, 2001; Yamaguchi and Hirano, 2006; Yoshida and Nagato, 2011). It has been reported that several of them are subjected to epigenetic regulation in diverse physiological processes (Zhang et al., 2010). However, the molecular mechanisms of how these genes are regulated epigenetically to control floral organ fate are largely unknown yet. We found that the C-class gene OsMADS58 was ectopically activated in ccp1 plants. Moreover, its constitutive expression in transgenic plants led to the formation of ccp1-like palea, while the carpelloid tissue did not appear in the lemma. This suggests an antagonism mechanism of the repression of C-class genes in first-whorl organ and might support, to some extent, that the palea and lemma are not the same whorl organs (Ren...
et al., 2013; Yun et al., 2013). Further ChIP experiments revealed that the deposition level of H3K27me3, an important epigenetic repression mark of chromatin, was decreased dramatically at the OsMADS58 locus, consequently releasing OsMADS58 expression from repression. Our study thus reveals an epigenetic mechanism of a MADS-box gene involved in floral organ development. The same epigenetic regulatory mechanism most likely functions in other biological processes since the global H3K27me3 modification level changed in ccp1 mutant, which displays pleiotropic phenotypes besides the OsMADS58-mediated floral organ identity. Actually, EMF1 in Arabidopsis are involved in various vegetative and reproductive developmental processes and responses to environmental stimuli (Kim et al., 2010, 2012). We therefore propose a model for roles of CCP1 in palea identity and other developmental processes/hormone response (Figure 7c). However, how CCP1 regulates H3k27me3 modification needs to be further investigated with extensive molecular and biochemical experiments.

PcG-mediated gene silencing is a common mechanism to maintain stable repression of target genes in diverse organisms. PRC1 and PRC2 are two best-characterized PcG complexes in animals. PRC2 labels the targets through adding H3K27 methyl marks, and PRC1 reads the methylation marks and mediates chromatin compaction and inhibition of the transcription machinery (Cao et al., 2002; Czermin et al., 2002; Kuzmichev et al., 2002; Muller et al., 2002; Schubert et al., 2005; Schwartz and Pirrotta, 2008; Morey and Helin, 2010). Although three PRC2-like complexes, EMF2-PRC2, VERNALIZATION2(VRN2)-PRC2 and FERTILIZATION INDEPENDENT SEED2 (FIS2)-PRC2, have been identified in Arabidopsis (Chanvivattana et al., 2004; Schubert et al., 2005; Kim et al., 2012), the components of PRC1 in plants have not been well determined. It was reported that EMF1 interacted with MSI1 and AtRING1A/1B, two proteins regarded as the members of PRC2 and PRC1 in Arabidopsis, respectively (Calonje et al., 2008; Bratzel et al., 2010), implying the involvement of EMF1 in PRC activity. EMF1 is required for the repression of flower development genes such as AG during stages of vegetative development (Calonje et al., 2008). The emf1 mutant lacks rosette shoot development with dramatically reduced inflorescence and produced stigmatic papillae and carpelloid structures (Chen et al., 1997; Aubert et al., 2001). The rice ccp1 mutant also displayed defects in plant architecture and floral organs, and CCP1 has the conserved function in the maintenance of H3K27me3 levels at OsMADS58, whose expression is critical to proper floral patterning. Thus, we propose that CCP1 might also act as a component of or participate in PRC1 and/or PRC2 pathway in rice. In support of this, we also found that CCP1 could interact with rice homolog proteins of MSI1 and AtRING1A/1B by yeast two-hybrid assay (Figure S6), whose functions are worth further investigation.

It is interesting that the ccp1 mutant presents also carpelloid structures while does not exhibit severe phenotype as Arabidopsis emf1 mutant. Additionally, ccp1 only displays slightly earlier flowering by 3 days, not like osemf2 that causes obvious delay in paddy field (Yang et al., 2013), although osemf2 is also dwarf and develops abnormal palea (Luo et al., 2009). Thus, we propose that CCP1 and...
OsEMF2 might function coordinately and also differentially in specific developmental processes in rice. Further experiments are certainly necessary to determine whether CCP1 is indeed a component of rice PRC and to isolate other components of the PRC to understand the molecular mechanisms underlying the complex interaction and epigenetic regulation of target genes in diverse biological processes.

EXPERIMENTAL PROCEDURES

Plant materials and growth condition

The recessive mutant ccp1 was isolated from an M2 population of Zhejing27 (japonica) mutagenized with γ-ray (Yang et al., 2011). Plants were cultivated in the experimental stations under natural conditions both in Sanya (Hainan Island) and Hangzhou (Zhejiang Province) for morphological and physiological analysis. Transgenic rice plants were grown in the phytotron under the conditions of 12 h light (28 ± 2°C)/12 h dark (25 ± 2°C) and 70-85% relative humidity, or the isolated field in our research station (Shanghai).

Map-based cloning and generation of transgenic plants

To identify the CCP1 gene, the ccp1 mutant (japonica) was crossed with Zhenshan97 (indica) to generate an F2 mapping population with 2180 individuals. The CCP1 locus was mapped to chromosome 1 by PCR-based mapping using the molecular markers (Table S2). The genomic fragments of the predicted genes within this region in ccp1 were PCR-amplified, sequenced and compared with the wild-type sequence for mutation detection. To generate a complementation construct, the 3.17-kb full-length coding region of CCP1 was cloned into the rice expression vector 35S-C1301 with the 35S promoter (Zhu et al., 2004) to create 35S-CCP1 construct. The construct was then introduced into ccp1 calli by Agrobacterium tumefaciens-mediated transformation and six out of nine T0 lines showed successful complementation. To generate OsMADS58 overexpression lines (OsMADS58-OE), the 819-bp coding sequence of OsMADS58 was amplified and cloned into the vector 35S-C1301, and transformed into the model variety Nipponbare (japonica). For the generation of OsMADS58 RNAi plants (OsMADS58-RNAi), the pTK303 vector was used (Wang et al., 2004). Primers for the constructs are listed in Table S2. More than 20 independent transformants with similar phenotypes were obtained for each construct.

Promoter activity assay

A 3.6-kb promoter region of CCP1 was amplified and cloned into the vector PB101.1. The fusion fragment of the promoter and GUS was digested and inserted into pCAMBIA1300 to create CCP1::GUS reporter construct, which was then introduced into Nipponbare by Agrobacterium tumefaciens-mediated transformation to generate more than 10 independent transgenic lines. Different tissues and organs from transgenic plants were collected for histochemical assay of GUS activity. Primers used for amplifying the promoter regions are listed in Table S2.

Subcellular localization of CCP1

The CCP1-GFP fusion construct was generated by in-frame fusion of the full-length CCP1 cDNA with GFP in the vector 35S-C1301. Transient expression was performed in onion epidermal cells using a helium biologic device (Bio-Rad, http://www.bio-rad.com) and in N. benthamiana leaves by injecting Agrobacterium tumefaciens harboring the CCP1-GFP construct. Confocal laser-scanning were performed as described previously (Zhang et al., 2011).

Microscope observation

Scanning electron microscopy and half-thin resin sections were performed as previously described (Zhang et al., 2011). Briefly, plant materials were fixed in a solution of 5% (v/v) acetic acid, 45% (v/v) ethanol, and 5% (v/v) formaldehyde at 4°C overnight and dehydrated in a graded ethanol series. For scanning electron microscopy, the samples were critical-point dried in liquid carbon dioxide and coated with gold, followed by visualization with the scanning electron microscope (JSM-6360LV, JEOL, http://www.jeol.co.jp). For histological sections, samples were embedded in Poly/Bed812 resin Ted Pella, http://www.tedpella.com, and then cut into 2 μm sections and stained with toluidine blue. Sections were examined microscopically (BX51, Olympus, http://www.olympus-global.com) and photographed.

RNA extraction and expression analysis

Total RNAs were isolated from young panicles before heading and 3-week-old seedlings using Trizol reagent (Thermo Fisher Scientific, http://www.thermofisher.com) and treated with DNase I using the DNA-free kit (Thermo Fisher Scientific). First strand cDNA was synthesized using Superscript III reverse transcriptase (Thermo Fisher Scientific) according to the manufacturer’s instructions. The quantitative real-time PCR experiments were performed using the SYBR Premix Ex Taq kit (Takara, http://www.takara.co.jp) and the Mastercycler ep realplex detection system (Eppendorf, http://www.eppendorf.com). The results were analyzed by the comparative C_{t} method and normalized using the ACTIN expression level. Three biological and three technical replicates were performed for expression analysis. RT-PCR analysis was executed using total RNAs and ACTIN was used as controls. The primers for RT-PCR and real-time PCR are listed in Table S2.

Immunoblot and H3K27me3 chromatin immunoprecipitation

Crude nuclear extracts were extracted from 3-week-old seedlings and the palea of young panicles of wild-type and ccp1 mutant, and protein immunoblot assays were performed as described previously (Ding et al., 2007). Amount of histone H3 protein detected by specific antibody was used as loading control. For H3K27me3 ChIP assay, 3-week-old seedlings and palea of 8–10 cm panicle (1.0 g fresh weight) were harvested for isolating nuclear extracts, and EpiQuik™ Plant ChIP Kit (Epigentek, http://www.epigentek.com) and anti-trimethyl-Histone H3 (Lys27) (Millipore, http://www.chemdupont.com) were used for ChIP assays. Primers used for real-time PCR detection are listed in Table S2.

BR treatment

The wild-type and ccp1 mutant seedlings were grown in MS medium for 10 days and then transferred to mediums containing 0, 1 or 5 μM epibrassinolide (an active BR component) for another 3 days to observe the bend of lamina joint.

Yeast two-hybrid assay

The constructs for yeast two-hybrid analysis were generated using the vectors pGBK7 and pGAD7 (Takara) that express protein fusions to the GAL4 DNA-binding domain or transcriptional-activation domain, respectively. The full-length cDNA sequences of...
CCP1, Os04g37740 and Os03g42890 were amplified by specific primers (Table S2) and inserted into pGBK7 and pGAD77, respectively. Two-hybrid analysis was performed as previously described (Wang et al., 2008).

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AUTHOR CONTRIBUTIONS

Conceived and designed the experiments: ZH, DY, QL. Performed the experiments: DY, XZ, LZ, SY, LZ. Analyzed the data: DY, XZ, ZH. Wrote the paper: ZH, DY.

CONFLICT OF INTERESTS

The authors have declared that no competing interests exist.

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article.

Figure S1. Differences in agronomic traits between wild-type and ccp1 mutants.

Figure S2. BR response and expression of BR-related genes.

Figure S3. Protein sequence alignment of CCP1 and EMF1.

Figure S4. Transgenic OsMADS58-OE plants and OsMADS58-RNAi/ccp1 plants.

Figure S5. ChiP assay for histone methylation pattern of the OsMADS58 gene in paelea.

Figure S6. Interaction analysis for CCP1 protein by yeast two-hybrid assay.

Table S1. Percentage of observed phenotypic classes in ccp1 mutant plants in paddy field growing in different regions and heading in different dates.

Table S2. Markers and primers used in this study.

REFERENCES


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