A novel superior factor widely controlling the rice grain quality

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Synthesis of storage starch and protein accumulation is the main action of endosperm organogenesis in term of the economic importance of rice. This event is strongly disturbed by abiotic stresses such as high temperature; thus, the upcoming global warming will cause a crisis with a great impact on food production[1,2]. The enzymes for the protein storage and starch synthesis pathway should work in concert to carry out the organogenesis of rice endosperm[3–5], but the regulatory mechanism is largely unknown. Here we show that a novel regulatory factor, named OsCEO1, acts as the conductor of endosperm organogenesis during the rice grain filling stage. The physiological properties of *floury-endosperm-2* (*flo2*) mutants showed many similarities to symptoms of grains developed under high-temperature conditions, suggesting important roles of the responsible gene in sensitivity to high-temperature stress. Our map-based cloning identified the responsible gene for the *flo2* mutant, *OsCEO1*, which has no homology to any genes of known function. The *OsCEO1* belongs to a novel conserved gene family and encodes a protein composed of 1,720 amino acid residues containing a TPR (tetratricopeptide repeat) motif, which is considered to mediate a protein-protein interaction. The yeast two-hybrid analysis raised an unknown protein showing homology to a late embryogenesis abundant protein and a putative basic helix-loop-helix protein as candidates for the direct interactor for OsCEO1, whereas no enzyme genes for the synthesis of storage substances were detected. The *flo2* mutant exhibited reduced expression of several genes for putative regulatory proteins as well as many enzymes involved in storage starch and proteins. These results suggest that *OsCEO1* is a superior conductor of the novel regulatory cascade of endosperm organogenesis and may have important roles in the response to high-temperature stress.

A comprehensive understanding of the mechanism of grain endosperm organogenesis may be necessary to solve the worldwide food crisis problem. Grain production activity is significantly sensitive to abiotic stresses, such as high or low temperature and submergence or desiccation. In particular, deleterious effects on the yield and quality of crop products, such as small grain size and low milling quality, are caused by high-temperature stress during the grain filling stage[1]. Yamakawa *et al*. reported that many features are altered in rice grains developed under high-temperature stress[6]. It is assumed that high-temperature stress affects many kinds of molecular mechanisms involving some regulatory systems. There are rice endosperm mutants showing obvious grain features that resemble those harmed by high-temperature stress[7,8]. Among them the *floury-endosperm-2* (*flo2*) mutation is unique because the multiple genes involved in storage starch biosynthesis are reduced, and therefore the responsible gene for the *flo2* mutation is considered to encode a regulatory factor[9].

We determined detailed characters of the *flo2* mutant. Grain size was significantly smaller compared with those of wild type (89% on average, $n > 40$, $p < 0.01$). These grains showed white and floury features, suggesting that the *flo2* mutant accumulated the aberrant storage starch (Fig. 1a and Supplementary Fig.1). Scanning electron microscopy (SEM) images of transverse sections of *flo2* grains indicated that the endosperm of the *flo2* mutant was filled with loosely packed starch granules composed of small granules with large air spaces, while the wild-type endosperm consisted of densely packed starch granules forming a big block per cell (Fig. 1b). The *flo2* mutant endosperm produced starch with a lower amylose content along with peculiar amylopectin, which lowered both the short and long chains, consisting of 9–21 degree of polymerization (DP) and ≥ 38 DP whereas increased middle chains with 22–38 DP (Fig. 1c). It is notable that grain developed under high-temperature condition resulted in aberrant starch in white grains with small size, as observed on the *flo2* mutant. Similar

to the *flo2* grains, a lower amylose content and peculiar amylopectin structure also occurs in these grains[6].

The *flo2* mutant exhibits severely decreased activities of branching enzyme 1 (BEI) along with a large number of enzymes involved in starch biosynthesis including starch branching enzymes (BEIIa, and BEIIb), ADP-glucose pyrophosphorylases (AGPS and AGPL) and soluble starch synthases (SS)[9]. Protein blot analyses detected an extreme reduction of BEI in the grain of the *flo2* mutant (Fig. 1d). The amylopectin properties of the BEI mutant resembled those of *flo2* grains, suggesting that the reduced BEI activity in the *flo2* mutant greatly influenced the structure of storage starch, although the BEI mutant bears seeds of normal size and normal-like features[10]. Likewise, mutations on other enzymes involved in starch biosynthesis show similar characteristics in part to those of the *flo2* mutant[8–12], but many differences are evident in their features. This fact demonstrates that the *flo2* mutation is a model of the ultimate feature caused by excessive high-temperature stress, and that the wild-type *Flo2* gene is involved in multiple events of endosperm organogenesis.

The responsible gene for the *flo2* mutation was identified by map-based cloning (Fig. 2a). The region of the gene was narrowed down to a 37 kb region on chromosome 4, and subsequent nucleotide sequence analysis revealed a G–A nucleotide substitution in the OsJNBa0070O11.2 gene in a *flo2* mutant. This mutation generated a stop codon in the predicted reading frame of the gene (Fig. 2b) and was detected in all tested F2 lines showing the recessive *flo2* phenotype with homozygous (Supplementary Fig. 2). We determined point mutations in another seven alleles of the *flo2* mutants (Fig. 2b). Furthermore, we confirmed the recovery of the *flo2* phenotype in transgenic lines harbouring the entire fragment of this gene of the wild type in the *flo2* mutant background. These lines set seeds of normal size and shape, formed rugged

cross-section endosperm surfaces, accumulated normal structured starch molecules, and restored the production of BEI (Fig. 1 and Supplementary Fig. 1). These results indicate that the loss of function of the gene invokes the phenotype of the *flo2* mutation. Thus, we named the responsible gene for the *flo2* mutation *Conductor of Endosperm Organogenesis 1* (*OsCEO1*).

There were homologous genes of *OsCEO1*, namely *OsCEO2* (CM000139-OsJ_06127, 59.8% similarity to OsCEO1) and *OsCEO3* (OSJNBa0084P08.1, 52.7% similarity to OsCEO1) in the rice genome. *OsCEO2* and *OsCEO3* were highly expressed in leaves, but poorly in immature seeds. Homologues to OsCEO1 were found in plant genomes, such as in *Arabidopsis* and grape, but none were found in any animals (Supplementary Fig. 3). These results suggest that *OsCEOs* constitute a novel conserved gene family in plants.

We determined the mRNA encoding the full-length predicted open reading frame (ORF), because the present database only registered a partial sequence for *OsCEO1* mRNA. The partial end sequence of four clones in the unidentified rice full-length cDNA pool indicated that these clones contained the full-length ORF (Supplementary Fig. 4). We sequenced them and confirmed that the perfect amino acid sequence was encoded for OsCEO1. We also confirmed the existence of the transcript in immature rice seeds by reverse transcriptase-mediated RNA-PCR (RT-PCR). The transcript of *OsCEO1* was abundantly detected in immature seeds and mature leaves but poorly detected in panicles before heading, stems and roots. During seed development, *OsCEO1* expression gradually increased as seed development progressed (Fig. 2c, d). This temporal expression may account for the appearance of the peculiar phenotype in the endosperm of the *flo2* mutant. *OsCEO1* expression was slightly higher just after suffering the high-temperature stress at 5 days after flowering (DAF), but then it rapidly

and significantly dropped after 10 DAF (Fig. 2d). Because the 5–15 DAF stage is most sensitive to high-temperature stress[6], this result suggests that *OsCEO1* expression is influenced by high-temperature stress.

The *OsCEO1* gene encoded a protein, in the middle of which there were three repeats of a tetratricopeptide repeat (TPR) motif (Fig. 2b). In other parts, no homology was found to any proteins with known function. The TPR motif is composed of tandem repeats of the 34 amino acid residues that adopt a helix–turn–helix structure and mediates protein–protein interactions[13,14]. To identify the interaction partners of OsCEO1, we screened the cDNA library of immature rice seed. Yeast two-hybrid screening raised many positive clones, and subsequent DNA sequencing showed that eight out of 29 clones encoded a gene for late embryogenesis abundant (LEA) proteins, and that seven of them corresponded to a putative basic helix–loop–helix (bHLH) protein (Fig. 2e and Supplementary Table 1). No clones encoded enzymes for the substance storage pathway, suggesting that the role of OsCEO1 is not as a direct regulator but rather as a global regulator. LEA proteins, constituing a large gene family in the rice genome, and bHLH proteins are suggested to be involved in a multiple stress response[15] and in the regulation of transcription[16], respectively, although their functions still remain unclear. Our results may suggest that these proteins are candidates interacting with OsCEO1 to construct a complex for the regulatory system.

We comprehensively analysed the transcripts expressed in the immature seeds at 10 DAF of the wild-type and *flo2* mutants using the microarray system established based upon the rice 8987 cDNAs[17], followed by confirmation with real-time quantitative RT-PCR for genes showing significant difference. The microarray analysis indicated that many genes were downregulated to less than half in the *flo2* mutants compared with those of the wild type (Table 1 and Supplementary Table 2). Expression

levels of many genes that participated in starch biosynthesis were significantly decreased, such as those previously reported[9]. Regarding storage protein accumulation, the expression of many genes encoding glutelin, globulin and 14–16 kDa allergenic proteins was strongly reduced. Protein blot analysis confirmed reduction of the 16 kDa allergenic protein (RA16) in the *flo2* mutant and restoration of its production in the transformant with the wild-type *OsCEO1* gene (Fig. 1d). Expression of heat-shock protein genes, such as HSP26, was not so altered in the *flo2* mutant, although it has been shown that high-temperature stress induces expression of these genes[6]. This fact indicates that heat-shock proteins are not directly involved in common symptoms of the *flo2* mutation and high-temperature stress. Gene expression for sucrose synthase (Susy1 and Susy2), pyruvate phosphate dikinase (PPDK), glucose-6-phosphate isomerase (PGIa), mitochondrial F1-ATPase, alanine amino transferase (AlaAT) and protein disulfate isomerase (PDI) was significantly decreased (Table 1). Susy is considered to be a key enzyme for the production of storage starch as well as cellulose synthesis[18]. PPDK has been reported as the responsible gene for the rice *flo4* mutant, which shows a similar appearance to the *flo2* mutant[19]. PGI is important for both glycolysis and gluconeogenesis by catalysing the reversible isomerization of glucose-6-phosphate to fructose-6-phosphate[20]. AlaAT is involved in the efficiency of nitrogen uptake and grain weight increase[21]. PDI is necessary for the precise sorting of storage proteins in protein bodies[22]. These results are similar to those observed in grain developed under high-temperature stress[6], suggesting a disruption of the common mechanism in the *flo2* mutant and by high-temperature stress.

The microarray analysis also demonstrated reduced expression of many genes that are experimentally unknown but expected to work as regulatory factors for transcription and signal transduction, such as a myb-domain protein, homologues to the CCAAT-binding protein, WRKY35, zinc-finger CCCH-domain protein and OsNAC6

and a protein kinase family protein. Reduced expression was found for a large number of genes for ATP synthesis and ribosomal proteins (Table 1). Decreased expression of some of these genes was also observed in grains developed under high-temperature stress. Thus, our results demonstrate that *OsCEO1* functions as a superior conductor for the novel regulatory mechanisms that govern the complex cellular system of endosperm organogenesis by controlling the individual downstream genes.

Method Summary

Detailed methods are described in the Supplementary Information. Raw data of the microarray analysis is available in the Rice Expression Database (http://red.dna.affrc.go.jp/RED/).

Plant materials and genome mapping. *Flo2* mutants were chemically mutagenized from a Japonica rice cultivar, Kinmaze, by N-methyl-N-nitrosourea[7]. The *flo2* locus was mapped using 641 F2 plants crossed between EM37 (*flo2*) and Kasalath (a wild-type Indica cultivar).

Complementation test. The fragment corresponding to the entire *OsCEO1* gene was cloned to pGWB1 (gifted by T. Nakagawa) by the Gateway LR recombination reaction system (Invitrogen, Carlsbad, CA, USA), which was introduced into the *flo2* mutant, EM37 using an *Agrobacterium*-mediated method[23].

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature

Acknowledgements

We thank K. Kadowaki for antibody; and K. Ono for technical assistance on generating transgenic plants. This work was support by grants from the Ministry of Agriculture, Forestry and Fisheries of Japan (Genome for Agricultural Innovation, MA2226, QTL-2006 and IPG-0022).

Author Contributions

H.Satoh created the *flo2* mutant; K-C.S., H.K., and T.A. determined characteristics of the mutant; K-C.S., M.F., T.A. and M.Y. mapped and cloned the responsible gene; K.K., S.K., N.K. and J.Y. performed microarray experiments and database arrangement; K-C.S., T.I., N.N., Y.T., K.M., E.I., characterized the OsCEO1; H.Y. and M.H. analysed grain starch; K-C.S., M.K., M.Y. and T.S. performed complementation of the gene; H.Shimada designed the study, analysed data and wrote the paper. K-C.S., H.K. and K.K. contributed equally to the study. All authors discussed the results and commented on the manuscript.

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Table 1 | Expression level of various genes in flo2 mutants.

	Gene Name*	Fold change (Average)†		
		EM37	EM36	H-Temp
Starch / Sucrose metabolism	AGPL	0.264	0.017	0.691
	AGPS	0.196	0.038	0.786
	BE1	0.018	0.002	1.056
	PGIa	0.413	0.301	0.697
	SS-I	0.189	0.140	0.965
	Susy1	0.423	0.260	0.774
	Susy2	0.290	0.142	1.046
Storage proteins	19K Globulin	0.018	0.002	
	Glutelin-A2	0.114	0.034	0.935
	RA16	0.121	0.008	
Putative transcription factor	CCAAT binding	0.041	0.012	0.971
	Myb1	0.123	0.020	
	OsNAC6	0.119	0.034	0.698
	WRKY35	0.046	0.031	
	zinc finger	0.053	0.017	0.997
Others	AlaAT-2	0.192	0.077	0.789
	ATPase β	0.288	0.259	0.624
	ATPase γ	0.048	0.003	1.027
	PPDK-B	0.334	0.040	0.448
	PDI	0.142	0.181	0.527
	protein kinase	0.058	0.014	1.036
	OsCEO1	0.717	0.451	0.829
	HSP26	0.852	0.711	6.725

The microarray values are means of the data obtained from independent hybridizations (n = 2). Detailed results are shown in Supplementary Table 2. No values are shown for several genes (blank column). Alteration of expression for each gene was confirmed by realtime quantitative RT-PCR (Supplementary Figure 5). *Simplified representation cited from the database or identified by similarity. †Average values of microarray signals on two *flo2* mutants (EM37 and EM36) and of grains that developed under high-temperature stress[6].

Figure 1 | Phenotype of the *flo2* mutant. a, The upper panel indicated the grain shapes of the wild type (WT), *flo2* mutant (EM37), vector control (VC), and complementary line (CP). Complementary lines and vector controls, which were transformed with/without a wild-type *OsCEO1* gene, were created from a *flo2* mutant, EM37. The lower panels show the images of the corresponding rice lines when they are illuminated with backlight, and the *floury* phenotype (EM37 and VC) is represented by a dark image. The WT and CP grains were transparent, and the EM37 and VC grains were chalky and floury. Scale bar = 1 cm. **b**, SEM image of WT, EM37, VC, and CP. Scale bar = 50 μm. **c**, Comparison of the chain-length profile of amylopectin. Debranched amylopectin was analyzed as described previously[6] and compared with those of WT. Difference in chain-length distribution of amylopectin is shown. **d**, Accumulation of BEI and an allergenic protein, RA16, in immature seed of 10 days after flowering (10 DAF) shown by protein blot analysis, using antiserum raised against BE1 and RA16, respectively. The RA16 antiserum detects highly conserved 14–16 kDa allergenic proteins with slightly different molecular weight. Proteins in seed from each of five CP lines and three VC lines were analyzed.

Figure 2 | Positional cloning of *OsCEO1*. **a**, Fine mapping of the *flo2* locus. The region of the *flo2* locus was mapped within 354 kb by published EST/SSR markers (RM3814 and RM3335-1), then narrowed to within a 37 kb region by newly created markers (218042 and 218787) in chromosome 4 (Chr. 4), which contained four predicted genes. **b**, Exon/intron structure of *OsCEO1*. The *OsCEO1* gene was composed of 23 exons (filled box) with 22 introns, including three tetratricopeptide repeat (TPR) motifs (open box) in the middle. Eight mutant alleles of the *OsCEO1* gene contained a base substitution that generated a premature stop codon (EM36, EM37, EM139, EM280, and EM554), an altered

splicing site (EM373 and EM624) or an amino acid change (EM756). **c**, Expression level of *OsCEO1* in the flag leaf (FL), panicles before heading (P), 10 DAF immature seed (IS), root (R), leaf blade (LB) , and stem (S). Filled box: wild type. Open box: *flo2* mutant (EM37). **d**, Expression of *OsCEO1* during seed development at 3, 4, 5, 7, 10 and 15 DAF. Filled box: wild type. Hatched box: wild type treated with high-temperature stress after 5 DAF. Error bars show standard deviations. e, Yeast two-hybrid analysis of OsCEO1 with an LEA protein (acc. no. AK061818) (left) and a bHLH protein (acc. no. AK070651) (right). Growth of yeast cells expressing LEA or bHLH (left), OsCEO1 (centre) and LEA or bHLH along with OsCEO1 (right) on agar plates lacking tryptophan, leucine and histidine in the medium. EV shows introduction of the empty vector instead of the corresponding genes.

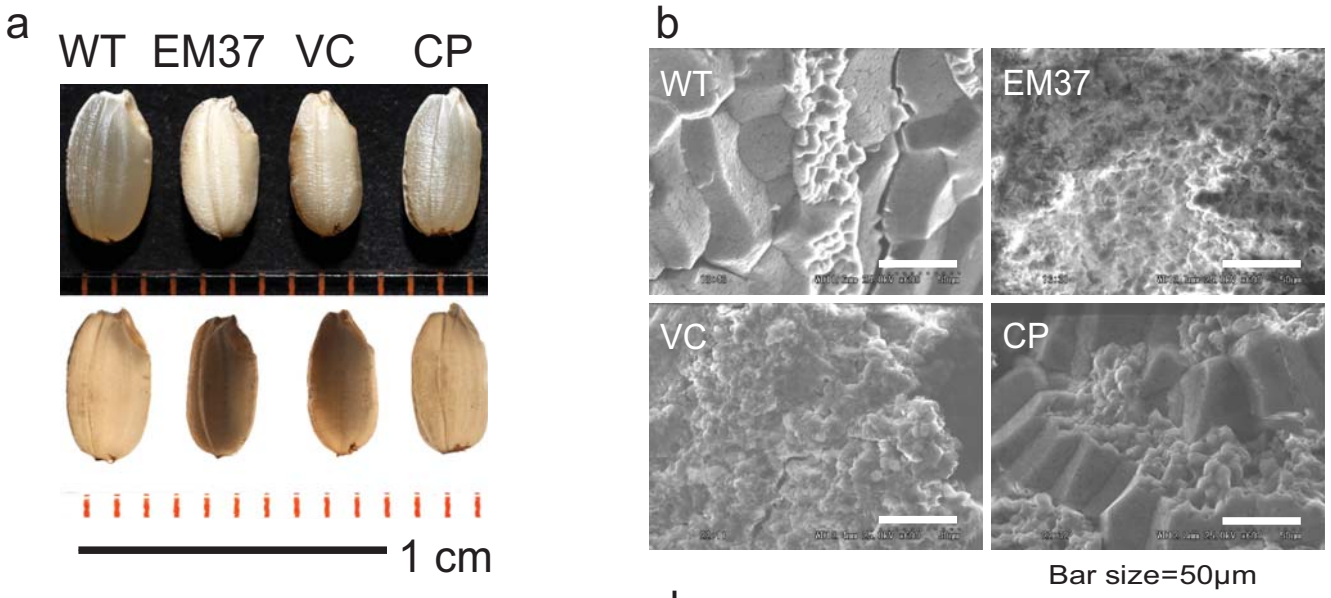

Figure 1

She et al. (H. Shimada)

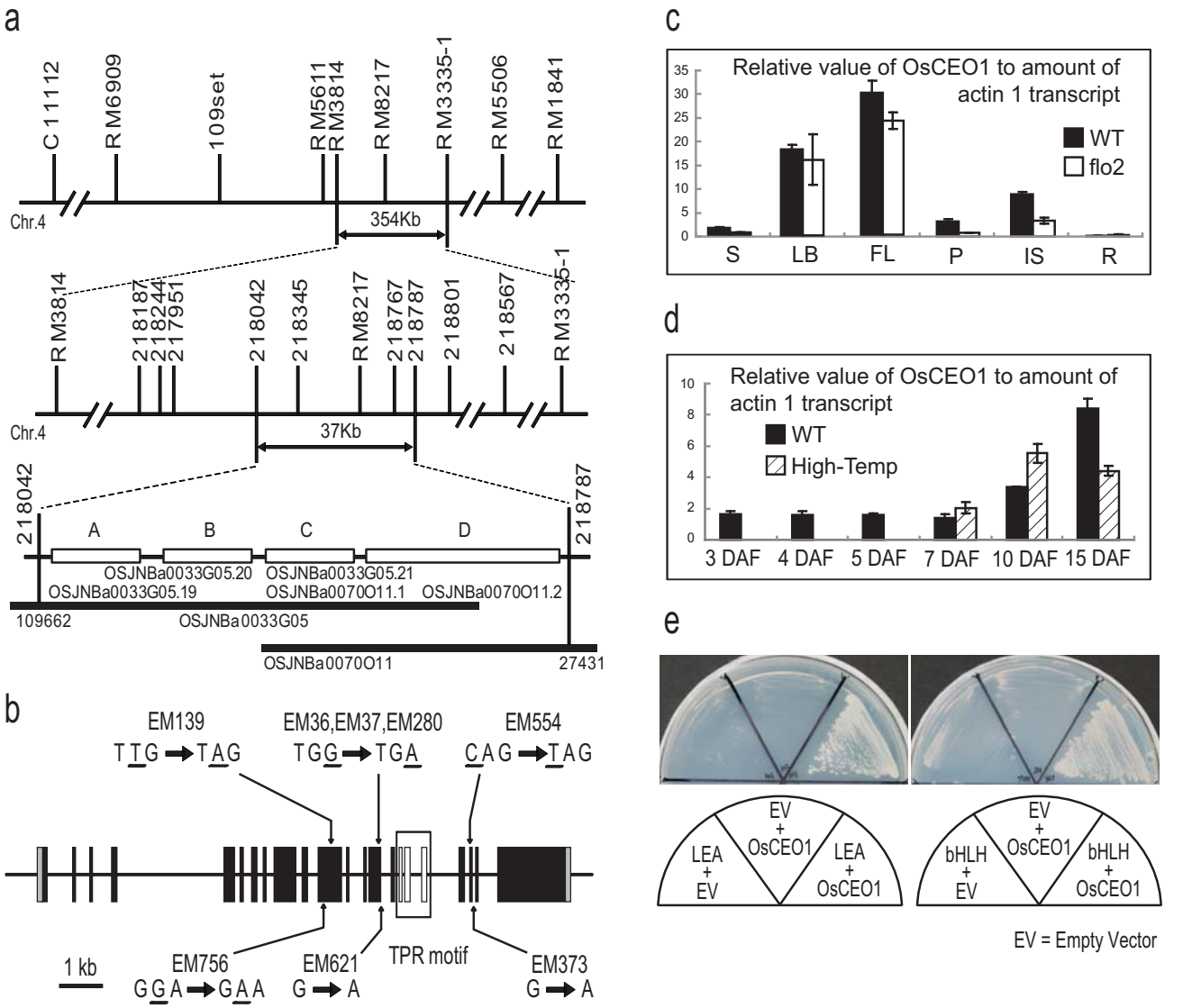

Figure 2

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