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Brief Communication

EARLY MORNING FLOWERING1 (EMF1) regulates the floret opening time by mediating lodicule cell wall formation in rice

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In the hybrid rice industry, the efficiency in F_1 seed production determines whether combinations can be widely used. In a traditional hybrid rice system, the restorer (R) line is the pollen donor, whereas the male sterile (MS) line is the pollen acceptor. The hybrid seed can be generated only if the floret opening time (FOT) of these two lines coincides. However, the average FOT of MS lines is usually later than R lines, especially in *indica-japonica* hybrid combinations, which greatly reduce hybrid seed yield. Yixiang 1A (YX1A) is an elite sterile line widely used in China, but its FOT is very late, resulting in low seed production in its different hybrid combinations, which not only increases the cost of hybrid seed production but also limits its further application.

In this study, we screened an early flowering mutant from the ethyl methanesulfonate mutagenized population of Yixiang 1B (YX1B), the corresponding maintainer line of YX1A. The mutant, *early-morning flowering1* (*emf1*), showed a ~2.5 h earlier flowering than its wild-type (WT), YX1B (Figure 1a and Figure S1). Lodicule is an important organ that controls the opening and closing of rice spikelets (Wang *et al.*, 1991). At the maximum flower opening angle, the area of the *emf1* lodicule was significantly larger than WT (Figure 1b). Through water absorption experiments, we found that the lodicule of *emf1* absorbs more water and expands quickly compared to WT (Figure 1c-d). Transmission electron microscopy revealed that the cell wall of lodicule of *emf1* was more loosen than that of WT (Figure 1e). Pectin, cellulose and hemicellulose, the main components of the cell wall, were significantly reduced in *emf1* (Figure 1f-i). Presumably, a decrease in lodicule cell wall components resulted in the loosening of the cell wall, which improved water absorption and expansion of lodicules in *emf1*.

To identify the causal gene conferring *emf1* phenotype, we performed fine mapping and narrowed the candidate gene to a 50-kb region containing eight candidate genes (Figure S2A). Using MutMap, we identified a 14-bp deletion with a high SNP index, which caused a frameshift in *LOC_Os01g42520* (Figure 1b and Figure S2B). The 2-kb promoter and coding sequence fragment of this gene from WT was transferred into *emf1,* and the phenotype was restored in the positive transgenic plants (Figure S2C-D). Thus, *LOC_Os01g42520* is the causal gene-regulating *emf1* phenotype.

The *EMF1* gene encodes an unknown protein, which is predicted to contain a signal peptide and a DUF642 domain (Figure S3A). GUS staining and relative expression analysis showed that *EMF1* is a constitutively expressed gene, but preferentially expressed in anther, stigma and lodicule near flowering (Figure S3B-C). A study reported that DUF642 showed preferential expression in the plant cell wall (Xie and Wang, 2016), and consistently, the eGFP subcellular assay revealed that EMF1 protein is located in the cell wall (Figure 1k). Comparative transcriptome analysis at the near-flowering stage from *emf1* and WT lodicules revealed that differentially expressed genes were enriched in biological processes related to the cell wall and pectin synthesis (Figure S3D-E). Further relative expression analyses confirmed that many cell wall-building genes were significantly down-regulated in *emf1* (Figure S3F). Therefore, *EMF1* may regulate the FOT by participating in the synthesis of cell wall components.

Pectin is synthesized and esterified in the Golgi apparatus and secreted to the cell wall to be de-esterified by pectin methylesterase (PME). Consistent with Wang *et al*. (2022), the degree of pectin methyl esterification was higher, but PME activity was significantly decreased in lodicules of *emf1* than WT (Figure S4). We additionally demonstrated that when PMEs were knocked out, the FOT of rice was only 1 h and 20 min earlier (Wang *et al.*, 2022), which was significantly lower than that of *EMF1* knockout lines (2.5 h earlier). The gap in FOT reveals that *EMF1* may also have an additional pathway to regulate the *emf1* phenotype.

We found several other proteins interacting with EMF1 in immunoprecipitation (Figure S5), amongst them, *Os01g0944700/OsGLN2* is a characterized gene that participates in the development of rice flowers (Akiyama and Pillai, 2001). Yeast two-hybrid assay showed interaction of GLN2 and EMF1

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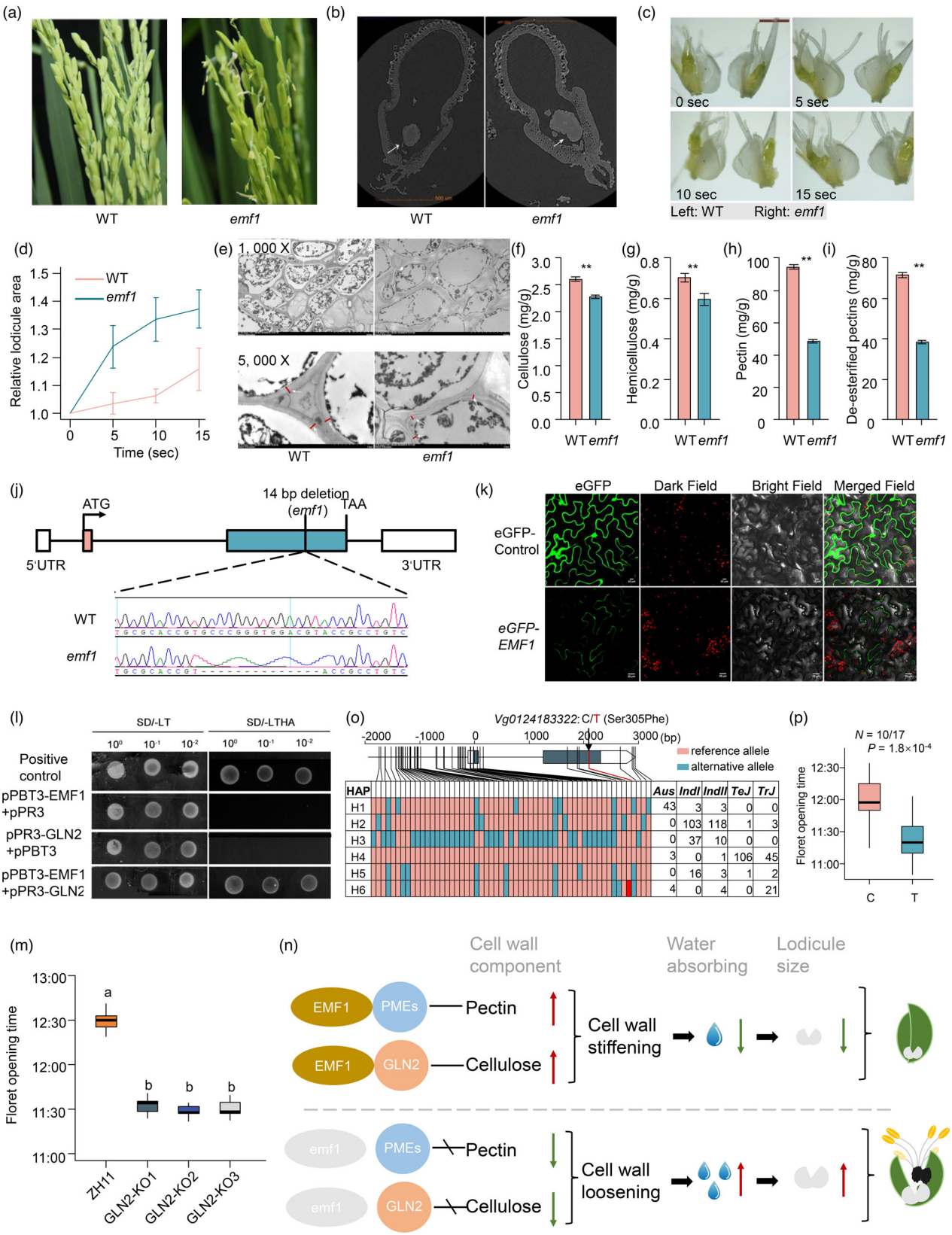

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Figure 1 The gene cloning, functional analyses and breeding application of *emf1*. (a) The earlier flowering phenotype of *emf1* compared to WT. (b) Cross-section of WT and *emf1* spikelet tomography. Scar bar, 500 μm. (c) Lodicule morphology of WT and *emf1* after water absorption. Scar bar, 1 mm. (d) Changes in WT and *emf1* lodicule surface area with time after water treatment. (e) The cell and cell wall morphology of lodicule of WT and *emf1* at maximum flowering angle observed using transmission electron microscopy. Scar bar, 10 and 2 μm below, respectively. (f-i) The cellulose (f), hemicellulose (g), pectin (h) and de-esterified pectin (i) contents in WT and *emf1*. (j) The gene structure and functional mutation of *EMF1*. (k) Subcellular localization of EMF1 protein in the cell wall. Scar bar, 20 μm. (l) EMF1 interacts with GLN2 in yeast cells. (m) The FOT of *OsGLN2* knockout lines. a, b indicate significant differences at $P < 0.01$. (n) A hypothesized model showing the molecular mechanism of EMF1 to regulate FOT in rice. (o) The haplotype analysis of *EMF1* in 533 diverse cultivated rice. (p) The FOT of *japonica* varieties with different alleles in the C/T variants in *EMF1*. $^{**}P < 0.01$. Significant differences were based on two-tailed *t*-tests.

(Figure 1l). To explore whether *EMF1* regulates FOT by interacting with GLN2, we developed transgenic knockout (KO) lines targeting *OsGLN2*. When *OsGLN2* was knocked out in cv. Zhonghua 11 (ZH11), the FOT of positive lines was ~1 h earlier than that of ZH11 (Figure 1m and S6A-C). It has been reported that expression of this fusion protein (OsGLN2-GST) in the prokaryotic system can specifically hydrolyze 1–3,1–6-β-glucanase from *Palmiform laminaria* (Akiyama and Pillai, 2001). To explore whether GLN2 affects FOT by regulating cell wall components as PMEs do, we measured cell wall components in *OsGLN2* knockout lines and ZH11. The contents of cellulose of KO lines were significantly lower than ZH11 (Figure S6F-H). Therefore, we speculated that *EMF1* regulates the content of pectin and cellulose in the cell wall by binding both PMEs and GLN2, thus affecting the water absorption of lodicule, which ultimately regulates FOT (Figure 1n).

To explore the breeding potential of *EMF1*, we generated a YX1A-*emf1* line by crossing *emf1* with YX1A (Figure S7A-D). The YX1A-emf1 showed a 2–2.5 h earlier FOT than the YX1A. To test *EMF1* applications in *japonica*, we knocked out *EMF1* in ZH11 and consistently we observed ~2 h earlier flowering (Figure S7E-G). Actually, the favourable allele of *EMF1* may have already been used in *japonica* FOT improvement through artificial selection. Haplotype analysis of *EMF1* in diverse rice germplasms (Zhou *et al.,* 2017) showed a C/T transition (an amino acid flip) in the second exon (Figure 1o). This mutation formed a new haplotype (H6) in tropical *japonica* and showed a significantly earlier FOT than the major haplotype (H4) in *japonica* (Figure 1p). The long-range linkage disequilibrium (LD) block and slow decay of extended haplotype homozygosity (EHH) around *EMF1* in tropical *japonica* indicate the selection of the H6 haplotype (Figure S8).

In summary, *EMF1* interacts with OsGLN2 to regulate the content of cellulose in the cell wall of the lodicule in addition to the previous interaction between EMF1 and PMEs. The loss of *EMF1* function resulted in increased water absorption capacity of lodicule and earlier FOT of rice. Our study provides insights into the regulation of rice FOT and could improve the efficiency of hybrid seed production in desirable male sterile lines.

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Conflict of interest

The authors declare no conflicts of interest.

Author contributions

Investigation, P. X., T. W., Y. L.; formal analysis, T. W., H. Z., X. C.; data curation and conceptualization, A. A., H. Z.; resources, P. X., Y. T., Y. L., J. F., C. S., H. W.; visualization, T. W., H.Z.; writing, A. A., H. Z.; supervision, W. W., H. Z., X. W.; funding acquisition, X. F., Q. J., X. W. Field management, Y. L.

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Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Appendix S1 Supplemental materials and methods.

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