The rice *EP3* and *OsFBK1* E3 ligases alter plant architecture and flower development, and affect transcript accumulation of microRNA pathway genes and their targets

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**Summary**

ERECTA PANICLE 3 (EP3) and ORYZA SATIVA F-BOX KELCH 1 (OsFBK1) proteins share 57% and 54% sequence identity with the Arabidopsis F-box protein HAWAIIAN SKIRT (HWS). Previously we showed that EP3 is a functional orthologue of HWS. Here we demonstrate that OsFBK1 is another functional orthologue of HWS and show the complexity of interaction between EP3 and OsFBK1 genes at different developmental stages of the plant. qRT-PCR expression analyses and studies of *EP3-GFP* and *OsFBK1-RFP* promoter reporter lines demonstrate that although EP3 and OsFBK1 expression can be detected in the same tissues some cells exclusively express *EP3* or *OsFBK1* whilst others co-express both genes. Loss, reduction or gain-of-function lines for *EP3* and *OsFBK1*, show that *EP3* and *OsFBK1* affect plant architecture, organ size, floral organ transcript levels.

**Keywords:** rice, F-box proteins, flower development, microRNA pathway.

**Introduction**

Ubiquitin protein degradation is a regulatory mechanism that affects many cellular processes including cell cycle, embryogenesis, development, floral formation, hormonal signalling, responses to stress and immunity in plants (Reed, 2003; Zhang et al., 2019). The level of key regulator proteins that modulate these processes is controlled by the 26S proteasome, which recruits E1, E2 and E3 enzymes to attach ubiquitin to proteins fated for degradation (Collins and Goldberg, 2017). The E3 ligase enzyme confers the required specificity by binding to the target substrate and the activated ubiquitin E2 complex, which is polyubiquitinated and then targeted for degradation by the 26S proteasome (Sharma et al., 2016). In rice, 1,332 types of E3 ligases have been reported (Du et al., 2009). The SCF complex, a kind of E3 ligase, is composed of four subunits: S-phase-kinase-associated protein-1 (Skp1), Cullin (Cul1), RING-finger protein (Rbx1/Roc1) and F-box protein (Yu and Matouschek, 2017). The F-box protein confers the specificity for recognition of correct targets for degradation (Petroski and Deshaies, 2005). In rice, 687 F-box proteins have been identified and shown to have diverse expression patterns, suggesting a range of roles during plant growth and development in response to internal and external signals (Jain et al., 2007).

The ERECTA PANICLE 3/LARGER PANICLE (EP3/LP, *Os02g15950*) and ORYZA SATIVA F-BOX KELCH 1 (*OsFBK1/ Os01g47050*) are two of the 687 F-box reported genes from rice. EP3 and OsFBK1 proteins share in excess 50% amino acid sequence homology to the Arabidopsis HWS F-box protein (Borah and Khurana, 2018; Yu et al., 2015).

The *EP3* gene was first identified during the description of the erecta panicle 3 (*ep3*) mutant. The characteristic upright panicle phenotype of *ep3* is due to an increase in small vascular bundles number and by their wider parenchymal tissues in the peduncles. Seed production is reduced in *ep3* (Piao et al., 2009). In 2011, Li et al described the characterization of two allelic mutants: larger panicle-1 (*lp1*) and 2 (*lp2*), which have a robust plant architecture, and bigger panicles resulting in higher yields. The mapping of these mutants indicated that the affected gene was *Os02g15950* and these are allelic mutants to *ep3*. The mutant *ep3* has a diminished leaf photosynthetic capacity and stomatal conduc-
tance due to reduction in size of stomatal guard cells (Yu et al.,...
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In 2007, Jain et al., identified OsFBK1 during a genome-wide analysis of F-box proteins in rice, and they demonstrated that OsFBK1 expression is higher during panicle development and during the development of roots of 7-day-old seedlings. OsFBK1 plays a role in regulating responses to drought (Borah et al., 2017). OsFBK1 targets two proteins for degradation: ORYZA SATIVA CINNAMOYL-COA REDUCTASE (OsCCR14) and OsATL5. OsCCR14 is a protein involved in the synthesis of lignin in roots and anthers of rice (Borah and Khurana, 2018), and OsATL5, is an E3 ligase protein that interacts with OsCCR14 and affects its enzymatic activity (Borah et al., 2021).

HAWAIIAN SKIRT (HWS), an F-box gene from Arabidopsis, is a regulator of plant growth, boundary formation and flower development (González-Carranza et al., 2007). Loss- and gain-of-function lines from this gene are pleiotropic. Loss-of-function lines show floral sepal fusion (González-Carranza et al., 2007). This is a phenotype shared with the double mutant of cuc1/cuc2 [CUP-SHAPED COTYLEDON1 (CUC1) and 2 (CUC2)] and Pro35:164B ectopic lines for the microRNA gene MIR164B (Aida et al., 1997; Laufs et al., 2004; Mallory et al., 2004; Mallory and Vaucheret, 2006). HWS loss-of-function plants are bigger, have longer roots and produce bigger seeds (González-Carranza et al., 2007). HWS affects cell proliferation and controls size and floral organ number by indirectly regulating the accumulation of the transcripts of CUC1 and CUC2 (González-Carranza et al., 2017). HWS is involved in the microRNA pathway (Lang et al., 2018; Zhang et al., 2017). We have demonstrated that EP3 is a functional orthologue of HWS (Yu et al., 2015). We hypothesize that OsFBK1 is a functional orthologue of HWS and that EP3 and OsFBK1 have similar functioning mechanisms to the Arabidopsis HWS gene in the miRNA pathway, additional to these reported by the Khurana’s group.

Here we show that OsFBK1 is a second functional orthologue of the Arabidopsis HWS gene. With the aid of fluorescent reporter genes and using qRT-PCR, we describe the patterns of expression of EP3 and OsFBK1 at transcript level and at whole plant and organ level. We report the effect that loss- or gain-of-function lines for these genes has on plant architecture, flower and grain development and yield. We show that EP3 affects transcript levels of OsPri-MIR164, OsNAM1 and OsNAC1. OsFBK1 alters OsNAC1, but not OsPri-MIR164 transcript levels, suggesting that OsNAC1 may be indirectly affected by EP3 via OsMIR164, but also by an independent regulatory pathway where OsFBK1 may be involved. We show that transcripts of the rice CRD1/OsHST (Zhu et al., 2019), OsDCL and OsWAF1 reported microRNA biogenesis pathway genes are altered by EP3 and OsFBK1. We identify the identification of putative rice orthologues of DDL, SE and OsHASTY (named as CRD1 by Zhu et al., 2019). The transcript levels of these genes are altered by EP3 and OsFBK1 and likely to be via protein degradation of a target yet to be identified.

Results and discussion

OsFBK1 is a rice functional orthologue of the Arabidopsis HWS gene

EP3 is a functional orthologue of HWS (Yu et al., 2015). In 2007, Jain et al., reported 687 potential F-box proteins in rice. Of these, the closest homologue to EP3 is OsFBK1. OsFBK1 shares 57% and 54% sequence homology with the rice EP3 and Arabidopsis HWS proteins respectively. To determine if OsFBK1 is also a functional orthologue of HWS, the predicted OsFBK1 cDNA coding region (1.236 Kb) was cloned using cDNA from the Nipponbare ecotype and sub-cloned into the vector pBI101.2:HWSpro (González-Carranza et al., 2007). Columbia-0 (Col-0) WT and hws-1 mutant plants were transformed and flower phenotypic comparisons between transgenic plants and controls were carried out. Flowers of the hws-1 plants failed to shed their sepals, which remained fused throughout pod development and senescence. Flowers from transformed plants with pBI101.2:HWSpro:OsFBK1 in both the Col-0 (Figure S1C,G) and hws-1 (Figure S1D,H) backgrounds resembled those of the Col-0 WT plants, sepal fusion was absent (Figure S1B,F) and floral organ shedding was restored to similar levels to the Col-0 WT (Figure S1A,E). Our results show that OsFBK1 is a functional rice orthologue of HWS, suggesting that the role of OsFBK1 during rice development may be similar to that of HWS in Arabidopsis, and that HWS has undergone a process of duplication in rice.

EP3 and OsFBK1 transcripts accumulate in the same tissues

To establish if the expression pattern of EP3 is similar to that of OsFBK1, we analysed EP3 and OsFBK1 transcript levels in rice tissues using real-time PCR (qRT-PCR). Results revealed differences in transcript accumulation relative to the expression in roots. A statistically significant EP3 over-expression was observed in panicle (10–15 cm long), followed by stem, leaf and grain; with increases of 7, 5.5, 4- and 2-fold change respectively (Figure 1A). Relative to the expression in roots, a statistically significant OsFBK1 over-expression was observed in leaf, followed by panicle and stem with increases of 6-, 3- and 2-fold changes respectively. OsFBK1 expression in grains was lower than that observed in roots (Figure 1B). These findings show that EP3 and OsFBK1 are most highly expressed in stems, leaves and panicles compared to roots and OsFBK1 expression in grains is lowest in milking stage grains.

Our results confirm the expression reported for EP3 by Piao et al. (2009) and Li et al. (2011), but differ from those reported for OsFBK1 by Borah and Khurana (2018). It is possible that the discrepancies observed in our expression analyses and those of Borah and Khurana (2018) are due to the differences in growing conditions or due to the use of different varieties. In our studies, we used Nipponbare japonica, and Borah and Khurana (2018) used Pusa Basmati1 indica. These subspecies exhibit different genetics, morphologies and physiologies (Yang et al., 2014).

GFP and RFP reporter analyses reveal expression or co-expression of EP3 and OsFBK1

To determine the cellular expression of EP3 and OsFBK1 during plant development single and double reporter transgenic lines in Nip background were generated. Genomic regions of 2.686 Kb and 2.739 Kb containing the promoters and 5’UTRs of EP3 and OsFBK1 were fused to GFP and RFP reporter genes respectively. A double transgenic line was generated by transforming calli with EP3pro::GFP and OsFBK1pro::RFP constructs. Expression was examined in T0 double transgenic lines using fluorescence or confocal microscopy. Results showed expression of EP3pro::GFP and OsFBK1pro::RFP in cells of roots, stems, leaves, flowers and seeds (Figure 2). EP3pro::GFP was highly expressed in cells of primary and secondary roots, meristematic zones cells, root cap cells and epidermis, and its expression was observed both in cytoplasm and in nucleus. The expression of OsFBK1pro::RFP was detected at low levels in the nucleus of meristematic zone cells, epidermis and root cap cells (Figure 2A–C). Transversal sections
of leaf number six showed expression of EP3pro::GFP in cytoplasm of proto-phloem, stomata and bulliform cells; and in nucleus of proto-phloem and bundle sheath cells. Co-expression of EP3pro::GFP and OsFBK1pro::RFP seen as orange colour compared with the red fluorescence produced by chlorophyll, was observed in mesophyll cells (Figure 2D,E). In stems, co-expression of EP3pro::GFP and OsFBK1pro::RFP was observed in hypodermis, epidermis, nucleus and bundle sheaths cells (Figure 2F–H). Images from WT stems are included for comparison (Figure 2L–N). Confocal floral analyses revealed that EP3pro::GFP was expressed in both the nucleus and cytoplasm of anther filaments, stamen, styles and stigma. OsFBK1pro::RFP was present in tapetum cells and co-expression of EP3pro::GFP and OsFBK1pro::RFP was observed in anthers, in the joining tissue between the anther and the anther filament, in pollen grains and developing ovaries (Figure 2I–Q). Fifty percent of pollen grains showed GFP expression confirming a single copy of mRFP1 gene insertion in the primary transgenic plants (Figure 2P). OsFBK1pro::RFP expression was absent in early stages of pollen development, but present in mature pollen. These results confirm the EP3 and OsFBK1 expression observed in tissues analysed by qRT-PCR and reveal cellular and subcellular localization of expression or co-expression of EP3 and OsFBK1. These data demonstrate that EP3 and OsFBK1 are co-expressed in some tissues. Some cells exclusively express either EP3 or OsFBK1 suggesting these genes may act independently in some cells and interdependently in others. Our results show that EP3 and OsFBK1 genes are expressed both in the nucleus and in the cytoplasm of cells from meristematic zones, roots, stems, leaves, flowers and seeds. We have been unable to generate functional EP3 and OsFBK1 transgenic fusions lines containing GFP or RFP tags proteins. It is likely the GFP and RFP tags interfere with the functionality of these F-box proteins in planta.

Loss, reduction or gain-of-function lines for EP3 and OsFBK1 are functional

In the absence of HWS expression, Arabidopsis plants are more robust and produce bigger plant organs, including rosettes, roots and seeds (Gonzalez-Carranza et al., 2007). We hypothesized that the lack of expression of EP3 and OsFBK1 would have similar effects in rice and the opposite would be true when these genes are over-expressed. To test this hypothesis, to determine the effect of OsFBK1 in rice development, and to understand the synergism between EP3 and OsFBK1 in shaping plant architecture, we generated and studied EP3 and OsFBK1 loss/reduced and gain-of-function rice plants. Homozygous plants from ep3 (Piao et al., 2009), osfbk1RNAi_1, EP3OE, OsFBK1OE and ep3/osfbk1RNAi were created, identified and analysed in these experiments. To confirm EP3 and OsFBK1 transcript absence or accumulation in loss- and gain-of-function lines generated, we performed qRT-PCR in 10-15 day old panicle tissues from three F2 plants from our ep3 (Piao et al., 2009), osfbk1RNAi_1, EP3OE, OsFBK1OE and ep3/osfbk1RNAi lines each. The same plants used to perform these analyses were also used in the phenotypic analyses described later. Expression patterns of EP3 and OsFBK1 for these lines are included in Figure 5 to facilitate comparison with expression patterns of putative genes influenced by EP3 and OsFBK1 and described later in the paper. Significant 38- and 2.3- fold increases were observed in EP3 transcript levels of EP3OE and osfbk1RNAi lines respectively (Figure 5A). The ep3 and ep3/osfbk1RNAi lines showed a reduction in EP3 transcript but not a complete reduction. The ep3 mutation introduces an earlier termination codon likely to produce a truncated protein of 266 aa. The primers used in the qRT-PCR analyses are located before the stop codon introduced earlier. It is likely these primers are detecting transcripts of the truncated protein showing in our analyses as a reduced expression of EP3. We used three biological replicates of these experiments, and this may explain why a high SD is observed in ep3 and ep3/osfbk1RNAi lines. Transcript levels of OsFBK1 were increased in ep3 (P < 0.05) and OsFBK1OE (P = 0.001) by 3- and 27-fold respectively. Significant reductions of OsFBK1 transcript were observed in EP3OE, osfbk1RNAi and ep3/osfbk1RNAi of 0.3, 0.6 and 0.75 respectively (Figure 5B). These results confirm that our ectopic lines are over-expressing EP3 and OsFBK1 transcripts. Our osfbk1RNAi is likely to be a knockdown with reduction of OsFBK1 transcript of about 2/3 compared to WT Nip. OsFBK1 transcript is reduced in ep3/osfbk1RNAi.

We attempted to generate a double knockout from EP3 and OsFBK1. Although we were unable to completely silence EP3 and OsFBK1 expression, we reduced OsFBK1 expression by about two-thirds compared to the wild type (Figure 5A,B). Analyses of our double EP3 KO and OsFBK1 knockdown lines show that the
flowers of these plants display floral fusion and abnormal growths at the top of the stigma as do the single knockdown lines (Figure 4). Floral fusion has been observed in the hws-1 mutant from Arabidopsis (Gonzalez-Carranza, et al., 2007), suggesting that the function of HWS and EP3 and OsFBK1 during flower development may be conserved in Arabidopsis and rice.

**EP3 and OsFBK1 alter plant architecture**

Morphological studies of the ep3 (Piao, et al., 2009), osfbk1RNAi, EP3OE, OsFBK1OE and ep3/osfbk1RNAi lines were performed throughout the development of plants grown in soil except for root length analyses that were performed in plants growing in hydroponics (Figure 3). Plant height of homozygous plants for EP3 and OsFBK1 loss/reduced and gain-of-function was analysed at 14, 21, 60 and 90 days (Figure 3A,B). The ep3 mutant did not show differences at 14 or 21 days, but 60- and 90-day-old plants were taller than WT-Hya by about 10 cm. EP3OE plants were shorter than WT-Hya by 10–20 cm throughout development. The osfbk1RNAi line showed shorter plants than the WT-Nip at 14, 21 and 60 days. At 90 days, these plants were taller than WT-Nip by about 10 cm. OsFBK1OE plants were shorter than WT-Nip plants at 14 and 21 days, but not different at 60 and 90 days compared to WT-Nip. The ep3/osfbk1RNAi line, which was generated by transforming the ep3 mutant (Hya background) showed shorter plants at 60 days and higher plants at 90 days compared to WT-Hya (Figure 3A,B). Tiller number of 60-day-old plants from all homozygous lines was analysed. Only osfbk1RNAi plants showed reduced numbers of tillers—about half—compared to WT-Nip (Figure 3C). Leaf length and width of fully expanded flag leaves from 90-day-old plants from all homozygous lines were measured. The ep3 and EP3OE flag leaves were wider and narrower respectively, about 1 cm each, compared to WT-Hya (Figure 3D, F). Homozygous plants from all lines were grown in hydroponics and the length of their roots was measured at 14 and 21 days. Shorter roots were observed at 21 days in EP3OE compared WT-Hya and in osfbk1RNAi and OsFBK1OE compared to WT-Nip. No significant differences were observed in other lines or in 14-day old plants (Figure 3E,G).

Our results show that ep3 plants are taller and have wider leaves than the EP3 over-expressing plants. Plants with reduced transcript levels of OsFBK1 are smaller during the initial developmental stages and taller after 90 days. They also have reduced tiller numbers. Down-regulation of EP3 and OsFBK1 also produces smaller plants that recover at 90 days. EP3 and OsFBK1...
over-expression produces smaller plants, with OsFBK1 over-expression plants recovering as development progresses. Our results suggest a complex interaction between EP3 and OsFBK1 affecting plant architecture. We cannot rule out that these differences are due to the differences in background of the mutants used. This data reveals a time-related effect of EP3 and OsFBK1 during rice development.

Panicle, grain development and yield are affected by EP3 and OsFBK1

To determine if the changes in plant architecture observed in our lines are associated with seed production and yield, we analysed panicle, grains and yield from five plants from each line generated and their controls. Consistent with previous reports (Piao et al., 2009; Yu et al., 2015) mature stage panicles of ep3 mutant were significantly shorter than those of WT-Hya. Panicles of OsFBK1OE were also significantly shorter than WT-Nip. EP3OE, osfbk1RNAi and ep3/osfbk1RNAi were not different to WT controls. EP3OE and OsFBK1OE lines showed significantly less grains per panicle compared to WT controls (22 and 17 less grains respectively). No difference in grains per panicle was observed in ep3, osfbk1RNAi and ep3/osfbk1RNAi lines. Significant reductions in filled grain number per panicle was observed in all lines studied compared to WT controls. The ep3/osfbk1RNAi and EP3OE produced on average 4 and 10 filled grains compared to 44 grains produced by WT (Table 1A, Figure S2A).

Previous analysis of Arabidopsis seeds indicated that in the absence of a functional HWS gene, seeds are bigger. The opposite is true in gain of function mutants (González-Carranza et al., 2007). To determine if EP3 and OsFBK1 alter rice grain size in a similar manner we measured the length, width, area and weight of 100 grains from mature panicles of each genotype. Grains of ep3, EP3OE and ep3/osfbk1RNAi were shorter than WT-Hya. In contrast, grains of osfbk1RNAi and OsFBK1OE were longer than WT-Nip. Grains of ep3 were wider than WT-Hya, while grains of EP3OE and ep3/osfbk1RNAi were narrower. Grains of osfbk1RNAi were wider than WT-Nip. The width of grains from the OsFBK1RNAi line were no different to WT Nip. Grain areas of ep3,
**EP3** and **ep3/osfbk1** were smaller than WT-Hya, while grain areas of **osfbk1** and **OsFBK1** were bigger than WT-Nip. Grains of **EP3** and **ep3/osfbk1** were lighter than WT-Hya. Grains of **osfbk1** and **OsFBK1** were lighter or heavier than WT-Nip respectively. The grains from ep3 were not different to the control WT (Table 1B, Figure S2B). These data show that EP3 and OsFBK1 affect yield by influencing panicle architecture, grain size and weight. Our results show that both EP3 and OsFBK1 affect grain filling. The reduction of seed size in the EP3OE line is consistent with our findings in Arabidopsis, that is, when the HWS gene is ectopically expressed the seeds are smaller. Surprisingly, reducing the expression of OsFBK1 results in bigger but lighter grains, while over-expressing OsFBK1 results in bigger and heavier grains. When the expression of both EP3 and OsFBK1 are reduced, grains are considerably smaller and lighter than when the expression of only one of the genes is non-functional or down-regulated, or when the EP3 is over-expressed. These results suggest that EP3 and OsFBK1 act antagonistically to regulate grain size and weight.

The functionality of F-box proteins relies on their interaction with other proteins of the SCF complex through domains in their N terminus (Petroski and Deshaies, 2005), and in the substrate specificity conferred by their C-terminus domain (Zhang et al., 2019). Li et al. (2011) identified two mutants of the EP3 gene: the lp1 and lp2, which are more robust, vigorous and productive compared with WT plants. The differences we observed in our ep3 studies are possibly due to the position and nature of the mutations in each line. The ep3 mutation is located in the middle of the gene, outside the F-box domain, and introduces an earlier termination codon resulting in a truncated protein (Piao et al., 2009). The lp1 mutation generates a protein of the same size as the WT, but with a Serine to Proline amino acid substitution at position 472. The lp2 mutation generates a longer protein than the WT by 21 amino acids caused by deletion of two nucleotides and a frameshift (Li et al., 2011). It is possible that in the ep3 lines, the interactions of the shorter EP3 peptide with the SCF complex and its target(s) are compromised, not allowing the protein to function properly. Detailed deletion analyses and amino-acid residue specificity of EP3 and OsFBK1, outside of the scope of this study, would provide more information on the mechanisms regulating grain yield and plant architecture in rice.

**Table 1** Effect of EP3 and OsFBK1 on (A) panicle and (B) grain development

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Panicle</th>
<th>Grain</th>
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<tbody>
<tr>
<td></td>
<td>Length (mm) (change vs WT)</td>
<td>Total grain/panicle (change vs WT)</td>
</tr>
<tr>
<td>WT-Hya</td>
<td>16.2 ± 1.3</td>
<td>56.0 ± 6.3</td>
</tr>
<tr>
<td>ep3</td>
<td>12.6 ± 0.9*</td>
<td>55.8 ± 4.4</td>
</tr>
<tr>
<td>EP3OE</td>
<td>13.9 ± 1.1</td>
<td>34.2 ± 7.9**</td>
</tr>
<tr>
<td>ep3/osfbk1</td>
<td>15.5 ± 2.7</td>
<td>43.8 ± 11.6</td>
</tr>
<tr>
<td>WT-Nip</td>
<td>19.0 ± 1.0</td>
<td>59.2 ± 6.8</td>
</tr>
<tr>
<td>osfbk1**</td>
<td>16.5 ± 2.2</td>
<td>45.6 ± 3.7</td>
</tr>
<tr>
<td>OsFBK1DE</td>
<td>13.2 ± 0.3**</td>
<td>41.8 ± 4.0**</td>
</tr>
</tbody>
</table>

Data show average values followed by ± SD. N values: (A) n = 35, (B) n = 700. The ep3, EP3OE and ep3/osfbk1 lines are in Hya background, while osfbk1** and OsFBK1DE are in Nip background. * and ** show significant differences at P < 0.05 and P < 0.01 respectively, determined by Student’s t-test (A) and regression analysis (B).
Table 2: Effect of EP3 and OsFBK1 on (A) floral organ number, (B) spikelet and stigma size and (C) anther size and pollen viability

(A) Number of:

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Glumes (palea + lemma)</th>
<th>Stamens</th>
<th>Stigmas</th>
<th>Carpels</th>
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<tbody>
<tr>
<td>WT-Hya</td>
<td>2.00 ± 0.00</td>
<td>6.00 ± 0.00</td>
<td>2.00 ± 0.00</td>
<td>1.00 ± 0.00</td>
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<tr>
<td>ep3</td>
<td>2.05 ± 0.22</td>
<td>6.24 ± 0.65*</td>
<td>2.27 ± 0.45**</td>
<td>1.00 ± 0.00</td>
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<td>EP3OE</td>
<td>2.00 ± 0.00</td>
<td>6.21 ± 0.51</td>
<td>1.89 ± 0.31</td>
<td>1.11 ± 0.31</td>
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<tr>
<td>ep3/osfbk1</td>
<td>2.19 ± 0.45**</td>
<td>6.02 ± 0.12</td>
<td>2.28 ± 0.45**</td>
<td>1.00 ± 0.00</td>
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<tr>
<td>WT-Nip</td>
<td>2.00 ± 0.00</td>
<td>6.00 ± 0.00</td>
<td>2.00 ± 0.00</td>
<td>1.00 ± 0.00</td>
</tr>
<tr>
<td>osfbk1/RNAi</td>
<td>2.00 ± 0.00</td>
<td>6.00 ± 0.00</td>
<td>2.00 ± 0.00</td>
<td>1.00 ± 0.00</td>
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<tr>
<td>OsFBK1OE</td>
<td>2.18 ± 0.39**</td>
<td>6.10 ± 0.37**</td>
<td>2.06 ± 0.25</td>
<td>1.18 ± 0.39*</td>
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(B) Spikelet

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<th>Genotype</th>
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<th>Width (mm)</th>
<th>Area (mm²)</th>
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</thead>
<tbody>
<tr>
<td>WT-Hya</td>
<td>6.83 ± 0.09</td>
<td>3.16 ± 0.12</td>
<td>16.78 ± 0.54</td>
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<td>ep3</td>
<td>6.78 ± 0.09</td>
<td>3.69 ± 0.09**</td>
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<td>EP3OE</td>
<td>6.01 ± 0.16**</td>
<td>2.67 ± 0.15**</td>
<td>12.49 ± 0.54**</td>
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<td>ep3/osfbk1</td>
<td>6.92 ± 0.17</td>
<td>3.84 ± 0.14**</td>
<td>20.33 ± 0.89**</td>
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<tr>
<td>WT-Nip</td>
<td>7.05 ± 0.19</td>
<td>3.44 ± 0.08</td>
<td>19.07 ± 0.56</td>
</tr>
<tr>
<td>osfbk1/RNAi</td>
<td>7.17 ± 0.14</td>
<td>3.31 ± 0.22*</td>
<td>18.49 ± 1.05</td>
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<tr>
<td>OsFBK1OE</td>
<td>7.08 ± 0.13</td>
<td>3.38 ± 0.15</td>
<td>18.12 ± 0.63**</td>
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<table>
<thead>
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<th>Genotype</th>
<th>Length (mm)</th>
<th>Width (mm)</th>
<th>Area (mm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT-Hya</td>
<td>1.16 ± 0.11</td>
<td>0.67 ± 0.15</td>
<td>0.66 ± 0.21</td>
</tr>
<tr>
<td>ep3</td>
<td>1.25 ± 0.14</td>
<td>0.75 ± 0.16</td>
<td>1.16 ± 0.33**</td>
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<tr>
<td>EP3OE</td>
<td>1.05 ± 0.06</td>
<td>0.54 ± 0.08</td>
<td>0.47 ± 0.11</td>
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<tr>
<td>ep3/osfbk1</td>
<td>1.54 ± 0.25**</td>
<td>0.82 ± 0.18</td>
<td>1.02 ± 0.28*</td>
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<tr>
<td>WT-Nip</td>
<td>1.42 ± 0.14</td>
<td>0.83 ± 0.06</td>
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</tr>
<tr>
<td>osfbk1/RNAi</td>
<td>1.11 ± 0.13**</td>
<td>0.57 ± 0.13**</td>
<td>0.54 ± 0.12**</td>
</tr>
<tr>
<td>OsFBK1OE</td>
<td>1.43 ± 0.15</td>
<td>0.71 ± 0.11*</td>
<td>1.20 ± 0.38*</td>
</tr>
</tbody>
</table>

(C) Anther

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Length (mm)</th>
<th>Width (mm)</th>
<th>Area (mm²)</th>
<th>pollen Viability Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT-Hya</td>
<td>1.57 ± 0.11</td>
<td>0.36 ± 0.02</td>
<td>0.59 ± 0.04</td>
<td>93.2 ± 1.60</td>
</tr>
<tr>
<td>ep3</td>
<td>1.85 ± 0.19**</td>
<td>0.45 ± 0.02**</td>
<td>1.30 ± 0.11**</td>
<td>85.8 ± 1.70**</td>
</tr>
<tr>
<td>EP3OE</td>
<td>1.27 ± 0.20**</td>
<td>0.37 ± 0.04</td>
<td>0.46 ± 0.08**</td>
<td>70.9 ± 2.20**</td>
</tr>
<tr>
<td>ep3/osfbk1</td>
<td>1.82 ± 0.18*</td>
<td>0.45 ± 0.04**</td>
<td>0.65 ± 0.07</td>
<td>67.9 ± 0.46**</td>
</tr>
<tr>
<td>WT-Nip</td>
<td>2.01 ± 0.09</td>
<td>0.40 ± 0.02</td>
<td>0.71 ± 0.05</td>
<td>92.6 ± 1.10</td>
</tr>
<tr>
<td>osfbk1/RNAi</td>
<td>1.90 ± 0.18</td>
<td>0.40 ± 0.03</td>
<td>0.61 ± 0.08*</td>
<td>87.2 ± 0.59*</td>
</tr>
<tr>
<td>OsFBK1OE</td>
<td>2.19 ± 0.18*</td>
<td>0.47 ± 0.03**</td>
<td>0.80 ± 0.09*</td>
<td>82.4 ± 2.80*</td>
</tr>
</tbody>
</table>

Data show average values followed by ± SD. N values: n = 63, except for pollen viability where n = 21. The ep3, EP3OE and ep3/osfbk1/RNAi lines are in Hya background, while osfbk1/RNAi, OsFBK1OE are in Nip background. * and ** show significant differences determined by Student’s t-tests at P < 0.05 and P < 0.01 respectively.

respectively. The number of stigmas increased in ep3 and ep3/osfbk1/RNAi compared to WT-Hya while the carpel number was increased in OsFBK1OE compared to WT-Nip (Table 2A; Figure 4).

During flower dissections, we observed that the size of floral organs was affected in the lines studied. Spikelets of EP3OE were shorter, narrower and occupied less area than WT-Hya. In ep3 and ep3/osfbk1/RNAi spikelets were wider and occupied a bigger area than WT-Hya. In osfbk1/RNAi these organs were narrower than WT-Nip, but the area they occupied in OsFBK1OE was smaller than WT-Nip (Table 2B). About 6% of ep3 spikelets displayed an extra glume outside the lemma or palea while about 13% of the ep3/osfbk1/RNAi spikelets had two or more fused florets (Figure 4A,F,K,P,U,Z, AE).

The area occupied by stigmas of ep3 was bigger than WT-Hya. In contrast, stigmas of osfbk1/RNAi were shorter, narrower and occupied less area than WT-Nip. Stigmas of OsFBK1OE were narrower but occupied a bigger area to WT-Nip. Stigmas of ep3/osfbk1/RNAi were longer, and occupied a bigger area compared to WT-Hya (Table 2B, Figure 4D,E,I,J,L,N,O,S, T,X,Y,A,C,AD,AH,Al). In WT flowers, carpels began to elongate and start their development after successful fertilization (Yhosida and Nagato, 2011). However, in about 12% and 11% of flowers of OsFBK1OE and ep3/osfbk1/RNAi respectively, elongation of carpels was observed before the onset of anthesis. Around 16% of OsFBK1OE flowers contained vellum-like organs inside the flowers (orange arrows, Figure 4A,H). EP3OE, OsFBK1OE and ep3/osfbk1/RNAi flowers presented double carpels in about 11% and 17% of the over-expressing lines and the double ep3/osfbk1/RNAi line respectively (double ovary only shown for OsFBK1OE and ep3/osfbk1/RNAi Figure 4AC, AH).
Figure 4  

EP3 and OsFBK1 affect flower development and floral organ number and size. (A, F, K, P, U, Z, and AE) Closed flowers: just before anthesis showing palea (p) and lemma (l). (B, G, L, O, V, AA and AF) dissected flowers without palea and lemma to show the inner floral whorls: lodicules (lo), stamens (s) and carpels (c). (D, I, N, S, X, AC and AH) Anthers. (E, J, O, T, Y, AD and AJ) Carpels showing stigma (st), style (sy) and ovaries (o). (F, J, O, T, Y, AD and AJ) Close up view of the style and ovary junction. Phenotypes shown include: (A-E) WT-Hya, (F-J) ep3, (K-O) EP3OE, (P-T) WT-Nip, (U-Y) osfbk1RNAi, (Z-AD) OSFBK1OE and (AE-AJ) ep3/osfbk1RNAi, note the double flower in the right side of the single flower in AE. The ep3, EP3OE and ep3/osfbk1RNAi lines are in Hya background, while osfbk1RNAi, OsFBK1OE are in Nip background. Yellow, blue, pink, white and orange arrows show sterile glumes, rudimentary glumes, extra glume, abnormal growths at the top of the ovary and velum-like structures respectively. White numbers in G and L show anther number, white numbers in I, N, AC and AH show stigma number. Blue numbers in AC and AH show ovary number. Scale bars: 1 mm, except for (E, J, O, T, Y, AD and AJ) where the scale is 500 µm.

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Anthers of ep3 were longer, wider and occupied a bigger area compared to WT-Hya. EP3OE anthers were shorter and occupied a smaller area compared to WT-Hya. The pollen viability of ep3, ep3/osfbk1RNAi, and EP3OE was reduced compared to WT-Hya. The area occupied by anthers of osfbk1RNAi was smaller than WT-Nip. In contrast, anther length, width and area of OsFBK1^{OE} were larger than WT-Nip. OsFBK1^{OE} and EP3^{OE} pollen viability was reduced. Anthers of ep3/osfbk1^{RNAi} were longer and wider compared to WT-Hya and the pollen viability of ep3/osfbk1^{RNAi} was lower than WT-Hya (Table 2C, Figure 4B,C,G,H,L,M,Q,R,V). Pollen viability is hormonally regulated (Acosta and Przybyl, 2019). Intersections between phytohormone responses and the miRNA pathway have been previously reported (Liu and Chen, 2009). Analysing hormonal homeostasis in all our transgenic lines will increase our understanding of these interactions.

During our dissection analyses, we discovered that about 24% and 26% of the ep3 and ep3/osfbk1^{RNAi} flowers respectively presented an uncharacteristic growth at the top of the styles (Figure 4I,IA,AJ,AK). Additionally, chimeric organs were observed in ep3/osfbk1^{RNAi} flowers (white arrows, Figure 4AH). Ectopic anthers originating from the lodicule were also present in about 2% of flowers. Detailed anatomical analyses of these growths will allow us to determine the type of cells contained in them. Down-regulation of EP3 results in an increased number of stamens and stigmas, as well as bigger floral organs. Over-expressing EP3 does not significantly change floral organ number but results in smaller floral organs. Down-regulation of OsFBK1 does not alter floral organ number but results in smaller spikelets and bigger stigmas and anthers. Over-expressing OsFBK1 increases floral organ numbers and anther sizes but reduces spikelet sizes (Table 1A).

Our gain- and loss-of-function EP3 and OsFBK1 lines show increased numbers of glumes, stamens, stigmas and carpels, differences in floral organ sizes and reduction of pollen viability. These findings suggest that EP3 and OsFBK1 may influence or regulate the expression of floral homeotic genes from the ABCDE model in rice (Sugiyama et al., 2019; Yoshida and Nagato, 2011). Analyses of the expression of these genes and the generation of double KO lines would support this hypothesis. Careful analyses of flowers showed the presence of a tumour-like growth at the top of the styles as well as the presence of chimeric organs in some floral organs highlighting a possible a role for EP3 and OsFBK1 in floral meristem regulation. Detailed anatomical, genetic and proteomic analyses of these plants will allow a greater understanding of the role of EP3 and OsFBK1 in flower development and meristem regulation.

Our anther analyses for OsFBK1 are different to those reported by Borah and Khurana (2018). Their OsFBK1^{OE} lines have shorter anthers while they are significantly longer in their OsFBK1^{KO} lines. Our OsFBK1^{OE} and OsFBK1^{RNAi} lines showed longer and shorter anthers respectively. These differences may be due to the genotypes chosen, the cloning vectors used, the genomic areas selected to generate these lines, the areas in the genome where the insertions took place, and/or the environmental conditions used to grow the plants. Borah and Khurana (2018) used an indica (Pusa Basmati1) variety while we used a japonica (Nip). The vectors used by Borah and Khurana, 2018 are pB4NU (OsFBK1^{OE}) and pANDA (OsFBK1^{KO}), while we used pBRACT214 (OsFBK1^{OE}) and pBRACT207 (OsFBK1^{RNAi}) vectors. Both OsFBK1^{OE} were created using a 1.236 kb full cDNA fragment; the pB4NU vector is 14.117 Kb (Mukherjee and Khurana, 2018) and uses restriction digestion/ligation as a cloning method, while the pBRACT214 is

9.165 kb (Rooke et al., 2000) and uses Gateway technology. The selected regions for silencing the gene are different, Borah and Khurana (2018) generated their OsFBK1^{KO} lines with a 0.298kb segment from the 3' UTR region, while we use a non-conserved region of 0.349 Kb in the 5' end of the coding region. We have grown our plants in greenhouses or controlled chambers in the UK. If the expression and function of OsFBK1 is affected by stress, it is possible that the morphology of anthers and other plant parts may be affected. Analysing the effect of the vectors, and the environmental conditions in the expression of OsFBK1 may help explain the differences observed.

Our results support the hypothesis that EP3 and OsFBK1 effect floral organ development in rice and may be functional orthologues of the HWS gene in Arabidopsis (Gonzalez-Carranza et al., 2007, 2017). Results also suggest that EP3 and OsFBK1 act antagonistically to influence the regulation of floral organ morphology, plant growth, architecture, flower development, pollen viability and yield in rice by targeting for degradation proteins that remain elusive.

**EP3 and OsFBK1 modulate transcript levels of OsNAM, OsNAC1 and OsPri-MIR164**

We demonstrated that in Arabidopsis, HWS regulates size and floral organ number by modulating CUC1 and CUC2 transcript levels via MIR164 (Gonzalez-Carranza et al., 2017). The rice putative orthologue of the Arabidopsis CUC1 and CUC2 genes is OsNAM (Hibara and Nagato, 2005). In rice, there are six MIR164 (a–f) (Sunkar et al., 2008). Here we designed primers to detect and quantify all the Pri-MIR164 from the six genes (Table S1).

To determine if EP3 and OsFBK1 act in the same pathway as HWS to regulate size and floral organ number in rice, and to determine if the stress related gene OsNAC1 (ORZYA SATIVA NO APICAL MERistem 1) known to be regulated by OsMIR164 (Chang et al., 2021; Fang et al., 2014) is affected by EP3 and OsFBK1, we quantified transcript levels of OsNAM, OsNAC1 and of OsPri-MIR164 using qRT-PCR. Significant increases of 6- to 6.5-fold in the transcript levels for OsNAM, OsNAC1 and OsNAC2 in the OE lines were observed in the EP3^{OE} line (Figure 5C–E). This is expected and an over-expression of OsPri-MIR164 suggests little or no presence of mature OsMIR164 regulatory transcripts. Therefore, up-regulation of OsNAM and OsNAC1 transcripts is observed. A significant decrease in transcript levels of OsPri-MIR164 was observed in ep3 (Figure 5E), however no significant increase of transcripts for OsNAM and OsNAC1 was observed (Figure 5C, D), possibly because ep3 is not completely silenced (Figure 5A), and enough mature OsMIR164 is present to regulate the transcript levels of OsNAM and OsNAC1. Significant differences were observed in transcript levels of OsNAC1 in osfbk1^{RNAi} and in ep3/osfbk1^{RNAi}. However, the accumulation of OsNAC1 transcript levels in ep3/osfbk1^{RNAi} is greater than the sum of each independent ep3 and osfbk1^{RNAi} line, and instead a slight reduction in expression compared to ep3 is observed, suggesting that EP3 and OsFBK1 regulate OsNAC1 in an antagonistic manner (Figure 5D). No significant differences in OsNAM or OsPri-MIR164 transcripts were observed in OsFBK1^{OE}, ep3/osfbk1^{RNAi} and OsFBK1^{KO} lines. Transcript levels of OsNAC1 were not affected in OsFBK1^{OE} lines (Figure 5D). These results suggest that EP3, but not OsFBK1, positively affects the transcript levels of OsPri-MIR164 and OsNAM. Transcript levels of OsNAC1 are regulated both by EP3 and OsFBK1 and an accumulative effect, which appears to be antagonistic, can be observed in the ep3/osfbk1^{RNAi} line (Figure 5D).
Transcript levels of EP3, OsFBK1, OsNAM1, OsNAC1, OsPri-MIR164, OsDDL, OsDCL, OsSE, OsWAF and OsHST genes are affected in panicles (10–15 cm old from booting stage) from single, double and over expressor EP3 and OsFBK1 lines. RT-qPCR measurements of (A), EP3; (B), OsFBK1; (C), OsNAM; (D), OsNAC1; (E), OsPri-MIR164 (F) OsDDL; (G), OsDCL; (H), OsSE; (I), OsWAF, and (J) OsHST RNA levels in : WT-Hya, ep3, EP3OE, WT-Nip, osfbk1RNAi, OsFBK1OE and ep3/osfbk1RNAi. The ep3, EP3OE and ep3/osfbk1RNAi lines are in Hya background, while osfbk1RNAi, OsFBK1OE are in Nip background. Two sets of statistical analyses were done using WT Hya and WT Nip as controls for EP3 and OsFBK1 respectively. Stars indicate a significant difference in the mean at *P ≤ 0.05, **P ≤ 0.01 and ***P ≤ 0.001. Relative expression values represent the mean ± SD of three biological replicates from each sample (n = 21).
Here we demonstrate that EP3 positively influences transcript levels of OsPri-MIR164 and OsNAM, while OsNAC1 transcript levels are affected by both EP3 and OsFBK1. These results suggest the presence of an alternative pathway in rice that influences OsNAC1 transcripts independent of MIR164, where OsFBK1 acts. Alternatively, OsNAC1 may be a target for OsFBK1. A protein–protein interaction experiment is needed to prove this hypothesis. OsNAM has been reported to be involved in regulating secondary branching of panicles and the number of spikelets under drought conditions (Kumar et al., 2014). OsNAM is also involved in secondary leaf and panicle development (Chang et al., 2021). Our qRT-PCR results help explain the phenotypes observed in our EP3 transgenic lines. OsNAC1 is a rice stress-sensitive gene (Fang et al., 2008; Khong et al., 2008). It is possible that OsFBK1 and EP3 may indirectly affect the regulatory mechanisms used by rice to regulate stress responses.

**EP3 and OsFBK1 affect transcript levels of genes involved in miRNA biogenesis**

Our studies in Arabidopsis show that Hv5 alters transcript levels of genes from the microRNA pathway (Zhang et al., 2017). We hypothesized that EP3 and OsFBK1 affect transcript levels genes involved in this pathway in rice. To test this hypothesis, we used qRT-PCR to analyse transcript levels of putative orthologues in rice known to be involved in biogenesis of microRNAs (Figure 5F-J). These include OsDCL (Liu et al., 2005) and OsWAF (Abe et al., 2010). We also identified and generated primers for qRT-PCR of the putative rice orthologues of other genes likely to be involved in biogenesis and transport of miRNAs. We based our identification on sequence similarities to the Arabidopsis genes, previously reported to be involved in microRNA biogenesis. We named them following the Arabidopsis nomenclature. These include OsDCL (OsSgo0546600) (International Rice Genome Sequencing Project, 2005), OsSE (OsSgo0698595) (Kawahara et al., 2013; Sakai et al., 2013) and OsHST (OsSgo363900; Rice Full-Length cDNA Consortium et al., 2003); named by Zhu et al. (2019) as CROWN ROOT DEFECT 1 (CRD1). Significant increases in transcript levels of OsDCL, OsDCL, OsSE, OsWAF and CRD1/OsHST were observed in the EP3OE line (Figure 5F-J). Significant increases in transcript levels of OsWAF and CRD1/OsHST were observed in the osfk-1 (OsSE) line (Figure 5J). Significant decreases or increases in transcript levels of OsDCL (Figure 5F) and CRD1/OsHST (Figure 5J) respectively were observed in OsFBK1OE. These results suggest that EP3 and OsFBK1 affect transcript accumulation of microRNA biogenesis genes, their transport and/or their function in rice, likely by targeting a protein involved in the miRNA pathway.

Here we have identified the rice miRNA pathway putative orthologue genes OsDCL, OsSE and OsHST. Detailed analyses of these genes are necessary to confirm if indeed they perform similar functions as their orthologues from Arabidopsis in the rice miRNA pathway. Zhu et al., 2019 have demonstrated that in CRD1/OsHST 65% of miRNA levels are down-regulated, suggesting that CRD1/OsHST is necessary for proper miRNA accumulation. We have shown that transcript levels of OsDCL, OsSE and OsHST genes are altered in gain- and loss-of-function lines of EP3 and OsFBK1 as well as those of OsDCL (Liu et al., 2005; Song et al., 2012, 2013) and OsWAF1 (Abe et al., 2010; Yu et al., 2005) genes. The data support the hypothesis that EP3 and OsFBK1 are involved in the miRNA pathway in rice. More analyses are necessary to determine their mode of action in such pathway. Many of the phenotypes described in this study may be regulated by microRNAs. OsWAF1, together with SHOOT ORGANISATION1 (DICER-LIKE4 homologue), SHOOTLESS2 (RD26 homologue) and SHOOTLESS4 (ARGONAUTE7 homologue) have been suggested to play a role in lemma and palea development (Abe et al., 2010; Toriba et al., 2010; Yoshida and Nagato, 2011). It will be interesting to investigate if these genes are affected in our EP3 and OsFBK1 lines in future studies.

A question that arises from our investigations and the observations reported in the literature for OsFBK1 is whether EP3 and OsFBK1 are targeting the same or different proteins for degradation. Landry et al. (2012) demonstrated that regulation of G1 cyclin levels in yeast is controlled by the two SCF ubiquitin ligases Cdc4 and Gr1r in a redundant manner related to the localization of the target in the nucleus or in the cytoplasm. They suggest the possibility of Cdc4 and Gr1 sharing additional redundant targets in their role to control cell cycle progression. Borah et al. (2021) have demonstrated that OsFBK1 targets for degradation OsCCR14 and the E3 ligase OsATL53. Other possible targets of OsFBK1 are yet to be elucidated. We have demonstrated that Hv5 affects the microRNA pathway (Zhang et al., 2017) and we report that EP3 and OsFBK1 may also be involved in the microRNA pathway in rice. Many important regulatory processes in cells are dependent on this pathway and tight regulation with a degree of flexibility is necessary for the survival of plants. More studies are necessary to prove if the functional redundancy of EP3 and OsFBK1 is present in rice and to define the targets of these genes.

**Materials and methods**

Detailed methods for plant materials, and techniques are available in (Appendix S1). Arabidopsis and rice plants were grown in growth rooms, or glasshouses (rice) using compost, soil or hydroponics.

Primers used in this study are included in Table S1.

For complementing hws-1 with the coding region of OsFBK1, genomic DNA from Nip-WT rice 10-day-old seedlings was extracted. The predicted coding region was amplified using primer set Rice1For/Rice1rev and cloned in the construct PriHWS:GUS (Gonzalez-Carranza et al., 2007).

Promoter reporter, RNAi knock out, and over-expressing lines of EP3 and OsFBK1 were generated using Gateway® cloning technology (Hartley, 2003; Nakagawa et al., 2007). To generate EP3pro:GFP/OsFBK1pro:RFP plants, a simultaneous transformation of calli was performed as described by Nishiumma et al. (2006) and Zhou et al. (2003). EP3pro:GFP, OsFBK1pro:RFP, OsFBK1(RNAi) and OsFBK1(CRISPR) lines were generated using WT-Nip and WT-Hya calli respectively. ep3/osfbk1(RNAi) was generated using ep3 calli (WT-Hya).

For EP3 (WT-Hya) and OsFBK1 (WT-Nip) expression analyses, total RNAs from 50-day-old roots, stems, leaves, panicles (10–20 cm) and young grains (milking stage) were extracted. For expression analyses of OsNAM, OsNAC1, OsPri-MIR164, OsDDL, OsDCL, OsSE, OsWAF and OsHST total RNA was extracted from 10- to 15-cm-long panicles of WT, ep3, EP3OE and ep3/osfbk1(RNAi) (Hya) and of WT, osfbk1(RNAi) and OsFBK1(CRISPR) (Nip). Putative orthologues of OsDCL, OsSE and OsHST were identified by comparing Arabidopsis sequence genes from TAIR (Berardini et al., 2015), using a BLAST search in NCBI (Geer et al., 2010) or the rice genome annotation project (Kawahara et al., 2013) databases. Data analyses were performed using LightCycler® 480 Software 1.5 & Excel 365.

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EP3pro:GFP and OsFBK1pro:RFP root (7-day-old plants), stems and leaf tissue (45-day-old plants) samples were embedded in 8% (w/v) agarose LMP (Helena Biosciences) and sectioned using a Ci 7000 vibratome (Campden instruments). GFP was excited using a 488nm line of multi argon ion laser and visualized between 500 and 530 nm. A combination of 488nm line of multi argon ion laser and 543nm line of a helium-neon laser were used to excite mRFP1 and visualized between 590 and 650 nm.

Glasshouse plants from WT-Hya, and homozygous lines for EP3, EP3OE, WT-Nip, Osfbk1RNAi, OsFBK1OE and ep3/osfbk1RNAi growing at the same time and under the same conditions, were used to analyse plant height, tiller number, and flag leaf dimensions. The same lines growing in hydroponics (Murchie et al., 2005) were used to analyse root length.

GFP and RFP expressions in calli and plant material were detected using either inverted Leica TCS SP6 confocal or Fluorescence Leica (Leica MZ10F) microscopes. Seed and floral morphology studies were performed using a dissecting stereomicroscope (Zeiss Stemi SV6). All measurements from microscopic images were performed using Fiji ImageJ (Schindelin et al., 2012). qRT-PCR statistical analyses were performed using GenStat (17.1.0.14713). For other measurements, One Way ANOVA and Tukey HSD were done using Microsoft excel 2016 and GenStat (17.1.0.14713).

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Conflict of interest

The authors have declared that no conflict of interest exist.

Author contributions

Z.H.G.C. conceptualized the project, conceived and designed the experiments; administered the project and funds; and wrote the manuscript. R.S.B, E.H.M and Z.H.G.C. acquired funds for this project. R.S.B, E.H.M, K.A.P, and Z.H.G.C. proposed the methodology. R.S.B, E.H.M, J.A.R. and Z.H.G.C. supervised the research. All authors, edited, read and approved the final version of the manuscript.

References


Supporting information

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

Figure S1 Complementation of hws-1 sepal fusion by OsFBK1.

Figure S2 Panicles and seeds from loss- and gain-of-function lines from EP3 and OsFBK1.

Table S1 Primers used in this study.

Appendix S1 Supplementary methods.