Transcriptional repression of GIF1 by the KIX-PPD-MYC repressor complex controls seed size in Arabidopsis

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Seed size is a key agronomic trait that greatly determines plant yield. Elucidating the molecular mechanism underlying seed size regulation is also an important question in developmental biology. Here, we show that the KIX-PPD-MYC-GIF1 pathway plays a crucial role in seed size control in Arabidopsis thaliana. Disruption of KIX8/9 and PPD1/2 causes large seeds due to increased cell proliferation and cell elongation in the integuments. KIX8/9 and PPD1/2 interact with transcription factors MYC3/4 to form the KIX-PPD-MYC complex in Arabidopsis. The KIX-PPD-MYC complex associates with the typical G-box sequence in the promoter of GRF-INTERACTING FACTOR 1 (GIF1), which promotes seed growth, and represses its expression. Genetic analyses support that KIX8/9, PPD1/2, MYC3/4, and GIF1 function in a common pathway to control seed size. Thus, our results reveal a genetic and molecular mechanism by which the transcription factors MYC3/4 recruit KIX8/9 and PPD1/2 to the promoter of GIF1 and repress its expression, thereby determining seed size in Arabidopsis.

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Seed size is one of important agronomic traits that influences seed yield of crops, and elucidating the molecular mechanism underlying seed size control will help to improve seed yield. In flowering plants, a mature seed consists of the embryo, endosperm and seed coat. Seed development starts with double fertilisation. One sperm cell fuses with the egg cell to form the embryo, and another sperm cell fuses with the central cell to generate the endosperm. Thus, the embryo and endosperm are derived from the zygotic tissues. The seed coat, which envelops the embryo and the endosperm, is derived from maternal integuments1–3. Seed size is therefore coordinately determined by zygotic and maternal tissues. Several genes have been shown to control seed size in Arabidopsis thaliana, such as DAI1, ENHANCER OF DAI-1 (EOD3), SUPPRESSOR OF DAI-1 (SOD7), BIG BROTHER (BB/EOD1), UBQUITIN-SPECIFIC PROTEASE (UBP15) GRI-PACTING FACTOR 1 (GIF1), SAMBA, AUXIN RESPONSE FACTOR 2 (ARF2), ARABIDOPSIS G PROTEIN GAMMA SUBUNIT 3 (AGG3), APETAL 2 (AP2), KLU and IKU1/2,4,5. However, the genetic and molecular mechanisms underlying seed size regulation are complicated and still largely unknown.

Cell proliferation and cell growth coordinately determine the organ size during the plant organogenesis.6,7: GRI-PACTING FACTORS (GIF1/2/3), a group of transcriptional co-activators, interact with Growth-Regulating Factors (GRFs) to control leaf, flower, and root development by regulating cell proliferation and growth8–14. The gif1 single mutants produce significantly smaller leaves and flowers than wild-type plants resulting from reduced cell number in Arabidopsis, while overexpression of GIF1 results in large leaf size due to an increase of cell number8–11,13. In addition, GIF1 also recruits SWI/SNF chromatin remodelling complexes to its target genes that can be transcriptionally activated or repressed by GRFs.15 In rice, overexpression of OsGIF1 increases the size of leaves, stems, and grains, while loss-of-function of OsGIF1 leads to small plants16,17. In maize, gif1 mutants are dwarf with narrow leaves resulting from a less cell number18. GIFs are involved in many developmental processes, but the molecular mechanisms of transcription activation and inhibition of GIFs is unknown.

PEAPODs (PPD1/2), which belong to the TIFY class II protein family, control leaf development, seed growth and germination, hypocotyl elongation, stomata development and flowering time19–22. Suppression of PPD genes leads to big and dome-shape leaves resulting from prolonged cell proliferation. PPD1/2 interact with KINASE-INDBUCIBLE DOMAIN INTERACTING 8/9 (KIX8/9) and TOPLESS (TPL) to form a repressor protein complex, which controls the leaf development by influencing the expression of cell division-related genes21,22. The stability of KIX-PPD complex is regulated by an F-box protein STERILE APETAL (SAP) that acts as a part of the SKP1/Cullin/F-box E3 ubiquitin ligase complex22,23. SAP positively regulates organ growth by targeting the KIX-PPD complex for 26S proteasome-dependent degradation22,23.

Here, we find that the KIX-PPD complex controls maternal integument development and influences seed size by regulating cell proliferation and growth. KIX8/9 and PPD1/2 interact with transcription factors MYC3/4 to form the KIX-PPD-MYC complex in Arabidopsis. The KIX-PPD-MYC complex binds to the typical G-box sequence in the GIF1 promoter and represses its expression. Genetic analyses show that GIF1 functions as a downstream factor of the SAP-KIX-PPD-MYC signalling pathway to control seed size. Our results reveal a genetic and molecular mechanism by which the transcriptional repression of GIF1 by the KIX-PPD-MYC complex regulates seed size in Arabidopsis.

Results

The KIX-PPD complex represses seed growth. KINASE-INDBUCIBLE DOMAIN INTERACTING 8/9 (KIX8/9) and PEAPOD1/2 (PPD1/2) were previously reported to form a KIX-PPD complex and regulate leaf size by influencing cell proliferation in Arabidopsis thaliana21,22. Here, we found an important function of the KIX-PPD complex in seed size control. The kix8-1 plants exhibited larger seeds than wild-type (Col-0) plants (Fig. 1a, c). Seed weight of kix8-1 plants was also heavier than that of wild-type plants (Fig. 1d). The size of cotyledons usually reflects changes in seed size24–26. Consistent with this, cotyledons of kix8-1 were larger than wild-type cotyledons (Fig. 1b, e). By contrast, seed size and weight and cotyledon area in kix9-1 plants were similar to those in the wild type (Fig. 1a–e). The kix8-1 kix9-1 double mutant showed significantly larger and heavier seeds and bigger cotyledons than the kix8-1 and kix9-1 single mutant (Fig. 1a–e), indicating that KIX8 and KIX9 function redundantly to control seed size and weight in Arabidopsis.

Seed area and weight and cotyledon area in ppd2-1 plants were increased compared with those in wild-type plants, while seed area and weight and cotyledon area in ppd1-2 plants were comparable to those in wild-type plants (Fig. 1a–e). Because the PPD1 gene (AT4G14713) is close to the PPD2 gene (AT4G14720) in the chromosome, we could not isolate the ppd1-2 ppd2-1 double mutants. We generated ppd2-1 mutation in the ppd1-2 mutant background and ppd1-cr mutation in the ppd2-1 mutant background to obtain the ppd1-2 ppd2-cr and ppd1-cr ppd2-1 double mutants using the CRISPR-Cas9 technology, respectively (Supplementary Fig. 1)27. The ppd1-2 ppd2-cr and ppd1-cr ppd2-1 mutants had similar phenotypes (Fig. 1a–e). Seed area and weight and cotyledon area in ppd1-2 ppd2-cr and ppd1-cr ppd2-1 mutants were significantly increased compared with those in ppd1-2 and ppd2-1 single mutants (Fig. 1a–e), indicating that PPD1 and PPD2 function redundantly to control seed size and weight. The ami-ppd and ppd2-1 plants have been reported to produce large and curved leaves with more cell number19–21. Interestingly, we observed that ppd1-2 ppd2-cr and ppd1-cr ppd2-1 showed strong curvature of leaves (Supplementary Fig. 2a), indicating that ppd1-2 ppd2-cr and ppd1-cr ppd2-1 are strong alleles compared with ami-ppd. We measured the leaf area of 32-d-old Col-0, ppd1-2 ppd2-cr and ppd1-cr ppd2-1 plants and found that the third to eight leaves of ppd1-2 ppd2-cr and ppd1-cr ppd2-1 were smaller than those of wild type, but the tenth and eleventh leaves of ppd1-2 ppd2-cr and ppd1-cr ppd2-1 were larger than those of wild type (Supplementary Fig. 2b, c). Considering that ami-ppd leaves had more cells than Col-0 leaves, we further examined palisade cell size and number of ppd1-2 ppd2-cr and ppd1-cr ppd2-1 fifth leaves. The palisade cell size of ppd1-2 ppd2-cr and ppd1-cr ppd2-1 plants was smaller than that of wild-type plants (Supplementary Fig. 2d, e). By contrast, the palisade cell number in ppd1-2 ppd2-cr and ppd1-cr ppd2-1 leaves was higher than that in wild-type leaves (Supplementary Fig. 2e), consistent with higher cell number in ami-ppd and ppd2-1 leaves19–21. These results supported that ppd1-2 ppd2-cr and ppd1-cr ppd2-1 promote cell proliferation in leaves, but decrease cell expansion. These data also suggest a possible compensation mechanism between cell number and cell size in ppd1-2 ppd2-cr and ppd1-cr ppd2-1. This compensation phenomenon has been observed in several mutants28–30.

Considering that KIX8/9 and PPD1/2 function in a complex21,22, we investigated the effect of simultaneous disruption of KIX8/9 and PPD1/2 on seed size. Seed area and weight and cotyledon area in the kix8-1 kix9-1 ppd1-2 ppd2-cr quadruple mutant were similar to those of ppd1-2 ppd2-cr or ppd1-cr ppd2-1 double mutants (Fig. 1a–e) suggesting that KIX8/9 and PPD1/2 act in the common pathway to control seed size. Together, these
Fig. 1 The KIX-PPD complex acts maternally to control seed development. a, b The seeds (a) and 8-day-old seedlings (b) of Col-0, kix8-1, kix9-1, kix8-1 kix9-1, ppd1-2, ppd2-2, ppd1-2 cr, ppd1-2 cr ppd2-1, and kix8-1 kix9-1 ppd1-2 ppd2-2 cr plants. c–e The relative seed area (c, n = 100), 100 seed weight (d, n = 10), and cotyledon area (e, n = 30) of Col-0, kix8-1, kix9-1, kix8-1 kix9-1, ppd1-2, ppd2-2, ppd1-2 cr, ppd1-2 cr ppd2-1, and kix8-1 kix9-1 ppd1-2 ppd2-2 cr. Seeds from the third to seventh silique on the stem of six plants were used for analysis. Cotyledons from the 8-day-old seedlings were used for analysis. The relative seed area (F1 seed area) and F2 seed area of Col-0, Col-0/kix8-1 kix9-1 ppd1-2 ppd2-2 cr (C/kkpp), col-0/kix8-1 kix9-1 ppd1-2 ppd2-2 cr (kkpp/kkpp), and kix8-1 kix9-1 ppd1-2 ppd2-2 cr/kx8-1 kix9-1 ppd1-2 ppd2-2 cr (kkpp/kkpp) plants (n = 100). h Ovules of Col-0 and kix8-1 kix9-1 ppd1-2 ppd2-2 cr plants at 0 DAP (days after pollination). i–l The seed area (j), outer integument length (j), outer integument cell number (k), and outer integument cell length (l) of Col-0 and kix8-1 kix9-1 ppd1-2 ppd2-2 cr plants at 0, 2, 4, and 6 DAP (n = 33). Ovules and seeds from six siliques, which were from the fourth silique on the stem of six plants, were used for analysis. Scale bars, 0.5 mm (a), 0.2 cm (b), and 50 μm (h). Error bars represent ±SE. Different lowercase letters above the columns indicate the significant difference among different groups, one-way ANOVA P-values: *P < 0.05 and **P < 0.01.
results show that the KIX-PPD complex restricts seed growth in Arabidopsis.

The KIX-PPD complex acts maternally to regulate seed size. Seed size is determined coordinately by the growth of maternal and zygotic tissues. To investigate whether the KIX-PPD complex functions maternally or zygotically to control seed size, we performed the reciprocal crossing experiments between the wild type and kix8-1 kix9-1 ppd1-2 ppd2-2. As shown in Fig. 1f, the F1 seed area of Col-0 plants pollinated with the pollen of kix8-1 kix9-1 ppd1-2 ppd2-2 plants was similar to that of self-pollinated Col-0 plants, and the F1 seed area of kix8-1 kix9-1 ppd1-2 ppd2-2 plants pollinated with the pollen of Col-0 plants was comparable to that of self-pollinated kix8-1 kix9-1 ppd1-2 ppd2-2 plants. The size of Col-0/kix8-1 kix9-1 ppd1-2 ppd2-2 and kix8-1 kix9-1 ppd1-2 ppd2-2/Cr/Col-0 F2 seeds was similar to that of Col-0/Col-0 F2 seeds and smaller than that of kix8-1 kix9-1 ppd1-2 ppd2-2/kix8-1 kix9-1 ppd1-2 ppd2-2 F2 seeds (Fig. 1g). In addition, reciprocal crossing experiments showed that kix8-1 or ppd2-1 single mutation acts maternally to influence seed size (Supplementary Fig. 3). Together, these results indicate that the KIX-PPD complex regulates seed growth through the maternal tissue of mother plants.

As the integuments belong to maternal tissues in Arabidopsis, we investigated the development of the outer integuments in the wild type and kix8-1 kix9-1 ppd1-2 ppd2-2. The kix8-1 kix9-1 ppd1-2 ppd2-2 plants had bigger ovule area and longer outer integuments than wild-type plants at 0 DAP (days after pollination) (Fig. 1h–j). We then counted the number of cells in wild-type and kix8-1 kix9-1 ppd1-2 ppd2-2 outer integuments and found that kix8-1 kix9-1 ppd1-2 ppd2-2 outer integuments contained more cells than wild-type outer integuments (Fig. 1k). By contrast, the length of cells in kix8-1 kix9-1 ppd1-2 ppd2-2 outer integuments was similar to that in wild-type outer integuments (Fig. 1l). These results indicate that the KIX-PPD module restricts cell proliferation in outer integuments before fertilisation. We then examined cell number and cell length in wild-type and kix8-1 kix9-1 ppd1-2 ppd2-2 outer integuments at 2, 4 and 6 DAP, respectively. The outer integument cell number and cell length of kix8-1 kix9-1 ppd1-2 ppd2-2 were increased compared with those of the wild type at 4 and 6 DAP (Fig. 1k–l), thereby resulting in long outer integument and large seed size in the kix8-1 kix9-1 ppd1-2 ppd2-2 plants (Fig. 1i–j). These results indicate that the KIX-PPD module limits both cell proliferation and cell elongation in outer integuments after fertilisation. We further compared the effect of kix8-1 kix9-1 ppd1-2 ppd2-2 on cell proliferation during ovule and seed developmental processes. As shown in Fig. 1k, the KIX-PPD module restrains cell proliferation of the outer integument during both ovule and early seed developmental stages.

The formation of the KIX8/9-PPD1-2/MYC3/4 complex. PPD1/2 and 12 JAZ proteins belong to TIFY class II protein family. JAZ proteins usually interact with transcription factors to perform their functions, such as ENHANCER OF GLABRA 3 (EGL3), GLABROUS 3 (GL3), TRANSPARENT TESTA 8 (TT8), MYEOCYTOMATOSIS 2/3/4 (MYC2/3/4), MYB21/24, GLABRA 1 (GL1), PURPLE ACID PHOSPHATASE 1 (PAP1), ETHYLENE-INSENSITIVE 3 (ein3) and ETHYLENE-INSENSITIVE3-LIKE 1 (eil1)32–33. We therefore used the split luciferase complementation assays to test whether PPD proteins could interact with these transcription factors. We found that PPD1 and PPD2 interacted with MYC3 and MYC4 (Fig. 2a), but not with MYC2 and other transcription factors (Supplementary Fig. 4). By contrast, we did not detect the interactions between KIX8/9 and MYC2/3/4 in split luciferase complementation assays (Supplementary Fig. 5). The interactions between PPD1/2 and MYC3/4 were further verified by forster resonance energy transfer and fluorescence lifetime imaging microscopy analyses (FRET-FLIM). As shown in Fig. 2b, the CFP fluorescence lifetime of MYC3-CFP was significantly decreased by the PPD1-YFP or PPD2-YFP in N. benthamiana leaves. The CFP fluorescence lifetime of MYC4-CFP was also significantly decreased by the PPD1-YFP or PPD2-YFP in N. benthamiana leaves. The bimolecular fluorescence complementation assays also showed that nYFP-PPD1 and nYFP-PPD2 associated with cyFP-MYC3 and cyFP-MYC4, but not with the cyFP control (Supplementary Fig. 6). To determine whether PPD1/2 could directly interact with MYC3/4 in vitro, we performed pull-down analyses. As shown in Fig. 2c, GST-MYC3/4 bound to MBP-PPD1/2 in vitro, but not the MBP control.

To investigate whether PPD1/2 could interact with MYC3/4 in Arabidopsis, we generated 35S:Myc-PPD1 #8, 35S:Myc-PPD2 #5, 35S:GFPMYC2 #4, 35S:GFPMYC3 #12, and 35S:GFPMYC4 #8 transgenic lines. 35S:Myc-PPD1 #8, 35S:Myc-PPD2 #5, 35S:GFPMYC3 #12, and 35S:GFPMYC4 #8 transgenic lines produced small seeds (Supplementary Figs. 9 and 10), indicating that they are functional. We crossed 35S:Myc-PPD1 #8 and 35S:Myc-PPD2 #5 lines with 35S:GFPMYC2 #4, 35S:GFPMYC3 #12, and 35S:GFPMYC4 #8 lines to generate 35S:MYC3-PPD1;35S:GFPMYC2, 35S:MYC3-PPD1;35S:GFPMYC4, 35S:MYC2-PPD2;35S:GFPMYC2, 35S:MYC2-PPD2;35S:GFPMYC4, 35S:MYC3-PPD2;35S:GFPMYC3, and 35S:MYC3-PPD3;35S:GFPMYC4 plants, respectively. Co-immunoprecipitation analyses (co-IP) showed that Myc-PPD1/2 associated with GFPMYC3/4 but not with the GFP control (Fig. 2d), indicating that PPD1/2 and MYC3/4 form a complex in Arabidopsis. The interactions between Myc-PPD1/2 and GFP-MYC2 were not found by co-immunoprecipitation analyses in Arabidopsis (Supplementary Fig. 7).

Pull-down analyses showed that His-KIX8 could not directly associate with GST-MYC3/4 in vitro (Fig. 2e). Considering that KIX8/9 can interact with PPD1/2, we incubated His-KIX8 with MBP-PPD1/2 and GST-MYC3/4. When proteins were pulled down by Ni-NTA agarose, we detected the MBP-PPD1/2 and GST-MYC3/4 proteins (Fig. 2e), indicating that KIX8, PPD1/2, and MYC3/4 could form a complex in vitro. The associations of His-KIX9 with GST-MYC3/4 were not found in similar assays (Supplementary Fig. 8). To further investigate whether KIX8/9, PPD1/2, and MYC3/4 form a complex in Arabidopsis, we generated 35S:Myc-KIX8 #6 and 35S:Myc-KIX9 #14 transgenic lines that formed small seeds compared with the wild type (Supplementary Fig. 9), and crossed them with 35S:GFPMYC2, 35S:GFPMYC3 #12, and 35S:GFPMYC4 #8 plants to isolate 35S:Myc-KIX8;35S:GFPMYC2, 35S:Myc-KIX8;35S:GFPMYC3, 35S:Myc-KIX8;35S:GFPMYC4, 35S:Myc-KIX9;35S:GFPMYC2, 35S:Myc-KIX9;35S:GFPMYC3, and 35S:Myc-KIX9;35S:GFPMYC4 plants, respectively. Co-immunoprecipitation analyses revealed that Myc-KIX8/9 and GFPMYC3/4 exist in a complex (Fig. 2f). Together, these results indicate that KIX8/9, PPD1/2, and MYC3/4 form a KIX-PPD-MYC complex in Arabidopsis.
myc3 and myc4 single mutant (Fig. 3a–e), indicating that MYC3 and MYC4 function redundantly to regulate seed size and weight. In addition, overexpression of GFP-MYC3/4 fusion proteins driven by the CaMV 35S promoter in wild-type plants resulted in small and light seeds compared with the wild type (Supplementary Fig. 10), indicating that MYC3/4 limit seed growth in Arabidopsis.

To investigate whether MYC3/4 function maternally or zygotically to control seed size, we performed reciprocal crossing experiments between the myc3 myc4/4 function maternally or zygotically to control seed size, we performed reciprocal crossing experiments between the myc3 myc4
Fig. 2 KIX8/9, PPD1/2, and MYC3/4 form a complex in Arabidopsis. a Split luciferase complementation assays showing the interactions between PPD1/2 and MYC3/4. b The FRET-FLIM assays showing that MYC3/4 interact with PPD1/2. c Pull-down analyses showing the interactions between PPD1/2 and MYC3/4 in vitro. GST-MYC3 and GST-MYC4 were incubated with MBP-PPD1, MBP-PPD2, and MBP, respectively. Proteins were pulled down by MBP-Trap-A agarose beads and detected by Western blot with anti-GST, anti-MBP, or anti-His antibody.

Fig. 3 MYC3 and MYC4 act maternally to control seed development. a, b The seeds (a) and 8-day-old seedlings (b) of Col-0, myc3, myc4, and myc3 myc4. c–e The relative seed area (c, n = 100), 100 seed weight (d, n = 10), and cotyledon area (e, n = 30) of Col-0, myc3, myc4, and myc3 myc4. Seeds from the third to seventh silique on the stem of six plants were used for analysis. Cotyledons from the 8-day-old seedlings were used for analysis.

f–h The relative area of F1 seeds (f) and F2 seeds (g) from the F1/C, Col-0/mmc4/C (mm), myc3 myc4/Col-0 (mm/C), and myc3 myc4/myc3 myc4 (mm/mm) plants (n = 100). h Ovules of Col-0 and myc3 myc4 plants at 0 DAP (days after pollination). i–l The seed area (i), outer integument length (j), outer integument cell number (k), and outer integument cell length (l) of Col-0 and myc3 myc4 plants at 0, 2, 4, and 6 DAP (n = 33). Ovules and seeds from six siliques, which were from the fourth silique on the stem of six plants, were used for analysis. Scale bars, 0.5 mm (a), 0.2 cm (b), and 50 μm (h). Error bars represent ±SE. Different lowercase letters above the columns indicate the significant difference among different groups, one-way ANOVA P-values: *P < 0.05. **P < 0.01.
shown in Fig. 3f, the F₁ seed area of Col-0 plants pollinated with the pollen of myc3 myc4 plants was similar to that of self-pollinated Col-0 plants, and the F₁ seed area of myc3 myc4 plants pollinated with the pollen of Col-0 plants was comparable to that of self-pollinated myc3 myc4 plants. In addition, the size of Col-0/myc3 myc4 and myc3 myc4/Col-0 F₂ seeds was similar to that of Col-0/Col-0 F₂ seeds and smaller than that of myc3 myc4/myc3 myc4 F₂ seeds (Fig. 3g). These results indicate that MYC3/4 act maternally to control seed size.

We then examined the development of wild-type and myc3 myc4 outer integuments. myc3 myc4 plants had larger ovules and longer outer integuments than the wild-type plants at 0 DAP (Fig. 3h–j). The number of cells in myc3 myc4 outer integuments was increased compared with that in wild-type outer integuments, while the outer integument cell length of myc3 myc4 was similar to that of the wild type at 0 DAP (Fig. 3k, l). These results indicate that MYC3/4 limit cell proliferation in the ovules before fertilisation. We further examined cell number and cell length in wild-type and myc3 myc4 outer integuments at 2 and 4 DAP. The outer integument cell number and length of myc3 myc4 were significantly increased compared with those of the wild type at 4 and 6 DAP, thereby resulting in longer outer integument and larger seed size in the myc3 myc4 plants (Fig. 3l–j). These results indicate that MYC3/4 limit both cell proliferation and cell elongation in outer integuments after fertilisation, consistent with the role of the KIX-PPD complex. We further compared the effect of myc3 myc4 on cell proliferation during ovule and seed developmental processes. As shown in Fig. 3k, MYC3/4 restrict cell proliferation in the integuments during both ovule and early seed developmental stages.

The KIX-PPD-MYC complex represses GIFI expression. We previously reported the SAP-KIX-PPD signalling pathway has an important role in leaf size control22,23, and performed the RNA-seq analysis using the first pair of leaves of 9-day-old myc3 myc4 and ppd1-2 ppd2-cr seedlings. 149 genes with significantly changed expression were found in both myc3 myc4 and ppd1-2 ppd2-cr plants (Supplementary Data 1). One of them was the transcriptional coactivator GIFI (GRF-INTERACTING FACTOR 1), which has been reported to control the size of leaves, flowers, seeds, and cotyledons.8,9,11,13,37,38. The expression of GIFI was significantly upregulated in both myc3 myc4 and ppd1-2 ppd2-cr seedlings (Supplementary Data 1). We also found that expression levels of GIFI were significantly higher in kix8-1 kix9-1, ppd1-2 ppd2-cr, and myc3 myc4 siliques than those in wild-type siliques at 0, 2, and 4 DAF (days after flowering) (Fig. 4a). By contrast, expression levels of GIFI were decreased in the 2 DAF siliques of 35S:Myc-KIX8 #6, 35S:Myc-KIX9 #14, 35S:Myc-PPD1 #8, 35S: Myc-PPD2 #5, 35S:GFP-MYC3 #12, and 35S:GFP-MYC4 #8 plants compared with those of wild-type plants (Supplementary Fig. 11). In addition, the LUC activity of GIFIpro:LUC was significantly reduced by overexpressing Myc-KIX8/9, Myc-PPD1/2 and Myc-MYC3/4 in the Col-0 protoplast (Fig. 4b). These results indicate that the KIX-PPD-MYC complex represses GIFI expression. The KIX-PPD module limits leaf development by the repressor TOPLESS (TPL)21,22. Overexpression of Myc-TPL also reduced the LUC activity of GIFIpro:LUC in the Col-0 protoplast (Fig. 4b). Plant cis-acting regulatory DNA element analysis showed that there was a typical G-box sequence (5’-CACGTG-3’) at the -425 bp site in the 2 kb promoter region of GIFI (https://www.dna.affrc.go.jp/PLACE/?action=newplace) (Fig. 4c). MYCs and PPDs had been reported to associate with the G-box sequence to regulate target gene expression21,39. Furthermore, down-regulation of PPDs orthologs in legume Medicago truncatula and legume soybean leads to significant increases in expression of MtGIF1 and GmGIF140. These results imply that GIFI might be a target gene of the KIX-PPD-MYC repressive complex.

To test this possibility, we performed the chromatin immunoprecipitation-quantitative PCR (ChIP-qPCR) assays using the 1–4 DAF siliques of 35S:GFP, 35S:GFP-MYC3:myc3, and 35S:GFP-MYC4:myc4 plants. The 35S:GFP-MYC3:myc3 and 35S:GFP-MYC4:myc4 plants formed small seeds compared with the wild type, indicating GFP-MYC3 and GFP-MYC4 are functional (Supplementary Fig. 12). As shown in Fig. 4c, d, the fragment F₁ from the GIFI promoter containing the typical G-box cis-acting element in 35S:GFP-MYC3:myc3 and 35S:GFP-MYC4:myc4 plants was remarkably enriched compared with that in 35S:GFP plants. The fragment F₁ from the GIFI promoter was also significantly enriched compared with other fragments (F2–F4) without the typical G-box element and the negative control (a fragment of ACTIN7 promoter) in 35S:GFP-MYC3:myc3 and 35S:GFP-MYC4:myc4 plants, indicating that MYC3 and MYC4 associate with the GIFI promoter through the fragment F₁ in Arabidopsis. We then asked whether KIX8/9 and PPD1/2 could influence the associations of MYC3 and MYC4 with the GIFI promoter. In 35S:GFP-MYC3:myc3 kix8-1 kix9-1, 35S:GFP-MYC4:myc4 kix8-1 kix9-1, 35S:GFP-MYC3:myc3 ppd1-2 ppd2-cr and 35S:GFP-MYC4:myc4 ppd1-2 ppd2-cr plants, the enrichment of F₁ fragments was substantially decreased compared with that in 35S:GFP-MYC3:myc3 and 35S:GFP-MYC4:myc4 plants (Fig. 4d), indicating that KIX8/9 and PPD1/2 are required for MYC3/4 to effectively bind to the GIFI promoter. The fragment F₁ from the GIFI promoter in 35S:GFP-KIX8kix8-1, 35S:GFP-KIX9kix9-1, 35S:GFP-PPD1ppd1-2, and 35S:GFP-PPD2ppd2-1 plants also could be strongly enriched compared with that in the 35S:GFP plants, while it could not be enriched in 35S:GFP-KIX8; kix8-1 myc3 myc4, 35S:GFP-KIX9; kix9-1 myc3 myc4, 35S:GFP-PPD1ppd1-2 myc3 myc4, and 35S:GFP-PPD2ppd2-1 myc3 myc4 plants (Fig. 4d). These results indicate that MYC3/4 are required for KIX8/9 and PPD1/2 to bind to the GIFI promoter and repress its expression. Consistent with this, the LUC activity of GIFIpro:LUC was not reduced by the overexpression of Myc-KIX8/9, Myc-PPD1/2, and Myc-TPL driven by the CaMV 35S promoter in the myc3 myc4 protoplast (Fig. 4b).

To investigate whether MYC3/4 could directly bind to the G-box cis-acting element in the promoter of GIFI, we performed the electrophoretic mobility shift assays (EMSA). As shown in Fig. 4e–g, MBP-MYC3 and MBP-MYC4 bound to the biotin-labelled probe A from the GIFI promoter containing the typical G-box (5’-CACGTG-3’) but not to the mutated biotin-labelled probe A (A-m). The binding ability of MBP-MYC3 and MBP- MYC4 to the probe A was decreased by adding the biotin-unlabelled probe A. These results indicate that MYC3 and MYC4 directly bind to the GIFI promoter.

GIFI acts maternally to control seed size. The gifi plants (SALK_150407) produced smaller leaves, seeds, and cotyledons than wild-type plants (Fig. 5a–c, e, and Supplementary Fig. 13a), consistent with previous studies.8,10,11. The seed weight of gifi plants was also significantly lower than that of wild-type plants (Fig. 5d). In addition, the fertility of gifi plants was lower than that of wild-type plants (Supplementary Fig. 13b). By contrast, overexpression of GIFI (35S:GIFI) led to bigger and heavier seeds and bigger cotyledons than the wild type (Fig. 5a–e). These results indicate that GIFI is required for normal seed and other organs development.

To investigate whether GIFI functions maternally or zygotically to control seed size, we conducted reciprocal crossing experiments between the wild type and gifi. As shown in Fig. 5f,
the F1 seed size of Col-0 plants pollinated with the pollen of gif1 plants was similar to that of self-pollinated Col-0 plants, and the F1 seed size of gif1 plants pollinated with the pollen of Col-0 plants was comparable to that of self-pollinated gif1 plants. In addition, the size of Col-0/gif1 and gif1/Col-0 F2 seeds was similar to that of Col-0/Col-0 F2 seeds and bigger than that of gif1/gif1 F2 seeds (Fig. 5g). These results support that GIF1 acts maternally to control seed size.

We then investigated the outer integument cell number and cell length before and after fertilisation. The gif1 plants had shorter outer integuments with fewer and shorter cells than the wild type at 0 DAP (Fig. 5h, i). We further examined the outer integument cell number and cell length after fertilisation. The gif1 showed shorter outer integuments and smaller seeds than the wild type before 6 DAP (Fig. 5j). The outer integument cell number of gif1 was significantly decreased compared with that of...
Fig. 5 GIF1 acts maternally to control seed development. a, b The seeds (a) and 8-day-old seedlings (b) of Col-0, gif1, 3SS::GIF1 #4, and 3SS::GIF1 #7. c–e The relative seed area (c, n = 100), 100 seed weight (d, n = 10), and cotyledon area (e, n = 30) of Col-0, gif1, 3SS::GIF1 #4, and 3SS::GIF1 #7. Seeds from the third to seventh siliques on the stem of six plants were used for analysis. Cotyledons from the 8-day-old seedlings were used for analysis. Scale bars, 0.5 mm (c). The third to seventh siliques on the stem of six plants were used for analysis. Cotyledons from the 8-day-old seedlings were used for analysis. f, g The relative area of F1 seeds (f) and F2 seeds (g) from Col-0/Col-0 (C/C), Col-0/gif1 (C/g), gif1/Col-0 (g/C), and gif1/gif1 (g/g) plants (n = 100). h The relative seed area (f), outer integument length (j), outer integument cell number (k), and outer integument cell length (l) of Col-0 and gif1 plants at 0, 2, 4, and 6 DAP (n = 33). Ovules and seeds from six siliques, which were from the fourth to seventh on the stem of six plants, were used for analysis. Scale bars, 0.5 mm (a), 0.2 cm (b), and 50 μm (h). Error bars represent ±SE. Different lowercase letters above the columns indicate the significant difference among different groups, one-way ANOVA P-values: *P < 0.05 and **P < 0.01.

Partially overlapping expression of KIX-PPD-MYC. As KIX8, KIX9, PPD1, PPD2, MYC3, MYC4 and GIF1 function in a signalling pathway to regulate seed size, we asked whether they have the similar expression patterns during ovule and seed development. To test this, we generated the KIX8pro::KIX8-GFP, KIX9pro::KIX9-GFP, PPD1pro::PPD1-GFP, PPD2pro::PPD2-GFP, MYC3pro::MYC3-GFP, MYC4pro::MYC4-GFP, and GIF1pro::GIF1-GFP transgenic lines. As shown in Fig. 6 and Supplementary Fig. 14, KIX8, KIX9, PPD1, PPD2, MYC3, MYC4, and GIF1 expressed in the integument and chalazal region of ovules before fertilisation. As shown in Fig. 6, PPD1, KIX9 and GIF1 strongly expressed in the nuclei of outer integument cells before 2 DAP and became weak from 3 DAP during seed development. MYC4 strongly expressed in the nuclei of outer integument cells before 3 DAP and became weak from 4 DAP during seed development. PPD2, KIX8, and MYC3 expressed in the nuclei of outer integument cells before 6 DAP. As shown in Supplementary Fig. 14, PPD1, PPD2, KIX9, and GIF1 also expressed in the chalazal domain cells of seeds before 2 DAP and were not observed at 4 DAP. KIX8, PPD2, MYC3, and MYC4 expressed in the chalazal domain cells of seeds before 4 DAP. In addition, MYC3 and GIF1 expressed in endosperms before 4 DAP. These results indicate that KIX8, KIX9, PPD1, PPD2, MYC3, MYC4 and GIF1 have overlapping expression patterns during ovule development and possess partially overlapping expression patterns during seed development, supporting that they function in a common pathway to control seed size. Moreover, the GFP fluorescence in the epidermal cells of GIF1pro::GIF1-GFP::myc3 myc4 outer integuments was observed at 4 and 5 DAP, which was not observed in GIF1pro::GIF1-GFP plants (Fig. 6), supporting that MYC3/4 repress GIF1 expression.
GIF1 acts with the KIX-PPD-MYC module to control seed size. As the KIX-PPD-MYC complex associates with the promoter of GIF1 through MYC3/4 and represses its expression, we asked whether GIF1 act in a common pathway with the KIX-PPD-MYC module to control seed size. We crossed gif1 with myc3 myc4, kix8-1 kix9-1, and ppd1-2 ppd2-2 to generate gif1 myc3 myc4, gif1 kix8-1 kix9-1, and gif1 ppd1-2 ppd2-2, respectively. As shown in Fig. 7a–c, e, the gif1 mutation entirely suppressed the large seed and cotyledon phenotypes of myc3 myc4, indicating that gif1 is epistatic to myc3 myc4 with respect to seed and cotyledon size. Similarly, gif1 is also epistatic to myc3 myc4 with respect to seed weight (Fig. 7d). The large size of seeds and cotyledons of kix8-1 kix9-1 and ppd1-2 ppd2-2 plants was strongly but not entirely suppressed by the gif1 mutation (Fig. 7a–c, e), indicating that KIX8/9 and PPD1/2 are strongly but not entirely dependent on GIF1 to control seed size. Similarly, the gif1 mutation strongly but not entirely suppressed the seed weight phenotype of kix8-1 kix9-1 and ppd1-2 ppd2-2 plants (Fig. 7d). These genetic analyses indicate that GIF1 acts in a common pathway with the KIX-PPD-MYC module to control seed size and weight.

The ppd1-2 ppd2-2 and kix8-1 kix9-1 plants produced wider siliques than wild-type plants. By contrast, gif1 plants had narrower siliques than wild-type plants (Supplementary Fig. 15). The gif1 mutation entirely suppressed the wide silique phenotype of kix8-1 kix9-1 plants and strongly suppressed the wide silique phenotype of ppd1-2 ppd2-2 plants (Supplementary Fig. 15). In addition, the silique phenotype of gif1 myc3 myc4 was similar to that of gif1 (Supplementary Fig. 15). These genetic analyses indicate that GIF1 acts genetically with the KIX-PPD-MYC module to control silique development. The gif1 and ppd1-2 ppd2-2 plants had lower fertility than wild-type plants (Supplementary Fig. 16). The myc3 myc4 and kix8-1 kix9-1 had similar fertility to wild-type plants, but the fertility of gif1 myc3 myc4 and gif1 kix8-1 kix9-1 was similar to that of gif1 plants (Supplementary Fig. 16). Additionally, the gif1 mutation decreased the fertility of ppd1-2 ppd2-2 plants (Supplementary Fig. 16). All of the genetic analyses indicate that GIF1 acts with the KIX-PPD-MYC module to control multiple biological processes.

GIF1 and SAP function in a common pathway to control seed size. STERILE APETALA (SAP/SUPPRESSOR OF DA1, SOD3) acts as a part of the E3 ubiquitin ligase complex to control organ size by regulating the stability of the KIX-PPD complex. The sod3-1 mutants produce small leaves, while 35S::SAP plants have large leaves. We found that 35S::SAP plants also produced bigger seeds and cotyledons than the wild-type plants (Fig. 7a–c, e). Similarly, 35S::SAP plants produced heavier seeds than wild-type plants (Fig. 7d). The gif1 mutation strongly suppressed the large seed and cotyledon phenotype of 35S::SAP plants (Fig. 7a–c, e). The gif1 mutation also strongly suppressed the heavy seed phenotype of 35S::SAP plants (Fig. 7d). Additionally, 35S::SAP plants produced wider siliques than wild-type plants, while it was significantly suppressed by the gif1 mutation (Supplementary Fig. 15). Although 35S::SAP plants had similar fertility to wild-type plants, the fertility of 35S::SAP::gif1 plants was similar to that of gif1 (Supplementary Fig. 16). Moreover, the GIF1 expression was obviously higher in the 3 DAP siliques of 35S::SAP plants than that in wild-type plants (Supplementary Fig. 17). These data indicate that GIF1 acts as a downstream factor of SAP to control seed size.
Seed size is the key agronomic trait that greatly determines the grain yield of plants. Although several factors have been reported to affect seed size in plants, the genetic and molecular mechanisms that determine seed size remain elusive. In this study, we discover a genetic and molecular mechanism that the transcription factors MYC3/4 recruit the repressor complex KIX8/9-PPD1/2 to the promoter of GIF1 and repress its expression, thereby determining seed size in Arabidopsis.
Previous studies reported that PPD1 and PPD2 act redundantly to regulate leaf size and shape by influencing both the primary and the secondary mitotic arrest fronts.\(^{20,21,23}\) Considering that ppd2-1 had large seeds, while ppd1-2 did not obviously affect seed size, ppd1-2 ppd2-1 double mutant will help understand the role of PPD1/2 in seed size control. However, the PPD1 gene (AT4G14713) is close to the PPD2 gene (AT4G14720) in the chromosome, we could not isolate ppd1-2 ppd2-1 double mutant. We therefore generated the ppd2-cr mutation in the ppd1-2 mutant background and the ppd1-cr mutation in the ppd2-1 mutant background to obtain the ppd1-2 ppd2-cr and ppd1-1 ppd2-1 double mutants using the CRISPR-Cas9 technology, respectively (Supplementary Fig. 1).\(^{22}\) The ppd1-2 ppd2-cr and ppd1-cr ppd2-1 mutants produced larger and heavier seeds than ppd1-2 and ppd2-1 single mutants (Fig. 1a, c, d), indicating that PPD1 and PPD2 function redundantly to control seed size and weight. KIX8/9 interact with PPD1/2 to form a transcriptional repressor complex and control leaf size.\(^{21,22}\) However, it is unclear whether the KIX-PPD complex affects seed growth in Arabidopsis. Here, we found that kix8-1 kix9-1 ppd1-2 ppd2-1 plants produced significantly larger seeds than the wild type (Fig. 1a, c). Reciprocal crossing experiments showed that the KIX-PPD complex functions maternally to control seed size (Fig. 1f, g). Cellular observation indicated that the KIX-PPD complex predominantly represses cell proliferation and also slightly limiting cell elongation in the integuments (Fig. 3k, l). These results reveal that the KIX-PPD complex negatively regulates seed growth in Arabidopsis.

The transcriptional repressor complex usually interacts with the transcription factors to regulate gene expression.\(^{43,44}\) We found that PPD1/2 could directly interact with the transcription factors MYC3/4 in vitro and in vivo, but not interact with MYC2. Although MYC3 and MYC4 share lots of overlapping functions with MYC2, distinct functions among them have been reported. For instance, MYC3 and MYC4 recognise similar cis-acting sequences (i.e. G-box and its variants) to MYC2, while the DNA-binding affinity of MYC3 and MYC4 differs from that of MYC2. MYC2 and MYC4 but not MYC3 interact with the JAZ4 protein. The expression levels of VEGETATIVE STORAGE PROTEIN 2 (VSP2) and PLANT DEFENSIN 1.2 (PDF1.2), two of JA marker genes, are significantly different in myc2, myc3, and myc4 single mutants when treated with JA.\(^{33,34,45}\) We further reveal that KIX8/9, PPD1/2, and MYC3/4 can form a complex in Arabidopsis (Fig. 2). Like kix8-1 kix9-1 and ppd1-2 ppd2-1 mutants, myc3 myc4 mutants produced bigger seeds than the wild type (Fig. 3a, c), consistent with a previous study,\(^{36}\) further suggesting that MYC3/4 have the overlapped function with KIX8/9 and PPD1/2 in seed size control. Reciprocal crossing experiments indicate that MYC3/4 act maternally to limit seed growth. Cellular observations show that MYC3/4 influence both cell proliferation and cell elongation in the integuments, consistent with the role of the KIX-PPD complex in seed growth control. Therefore, the KIX-PPD-MYC module is crucial for seed size control in Arabidopsis.

To identify the targets of the KIX-PPD-MYC module in seed growth, we performed the RNA-seq and found that PPD1/2 and MYC3/4 repress the expression of GIF1. The expression levels of GIF1 in 0, 2, and 4 DAF siliques from kix8-1 kix9-1, ppd1-2 ppd2-cr and myc3 myc4 plants were significantly higher than those of wild-type plants (Fig. 4a). Consistent with this, overexpression of Myc-KIX8/9, Myc-PPD1/2, Myc-MYC3/4, and Myc-TPL could reduce the activity of GIF1pro::LUC (Fig. 4b). In addition, EMSA experiments showed that MYC3 and MYC4 directly bind to the G-box sequence in the promoter of GIF1 (Fig. 4f, g). ChIP-qPCR analyses showed that KIX8/9 and PPDP1/2 directly associate with the promoter of GIF1 through MYC3/4 (Fig. 4d). These findings indicate that MYC3/4 may recruit the transcriptional repressor complex TPL-KIX-PPD to the promoter of GIF1 to repress its expression (Fig. 7f). Overexpression of KIX8, KIX9, PPD1, PPD2, MYC3, or MYC4 led to small seeds (Supplementary Figs. 5 and 6), consistent with the result of PPDJOE (PPDJOE).\(^{19}\) It is possible that overexpression of KIX8, KIX9, PPD1, PPD2, MYC3, or MYC4 in Arabidopsis might have more probability to form the TPL-KIX-PPD-MYC complex that represses the expression of GIF1, thereby resulting in small seeds. Interestingly, MYC proteins recruit the TPL-NINJA-JAZ transcriptional repressor complex to regulate gene expression.\(^{43,46,47}\) Overexpression of JAZ13 alone attenuates JA-induced defence responses in Arabidopsis leaves. Overexpression of NINJA promotes root length when treated with MeJA.\(^{43}\) Overexpression of MYC2, MYC3, or MYC4 accelerates JA-induced leaf senescence.\(^{48}\) These results indicate that overexpression of the single complex components can cause phenotypes. The expression of GIF2 and GIF3 in the 3 DAP siliques of kix8-1 kix9-1, ppd1-2 ppd2-cr, and myc3 myc4 plants was also upregulated compared with that in the wild type (Supplementary Fig. 18). Down-regulation of PPDs orthologs in legume Medicago truncatula and soybean leads to the high expression of MtGIF1 and GmGIF1 in leaves, stipules, and seeds, respectively.\(^{49}\) These results indicate that the expression of GIFs regulated by PPDs might be a common mechanism in dicotyledon plants. The loss-of-function mutation in GIF1 produced smaller seeds and cotyledons than the wild type (Fig. 5a–e), consistent with previous studies.\(^{10,11}\) The gif1 mutant has fewer cells and longer cells in the integuments than the wild type (Fig. 5k, l), suggesting a compensation mechanism between cell proliferation and cell elongation. This phenomenon has been observed in several seed size mutants.\(^{28,31,42}\) In addition, GIF1 was reported to play significant roles in leaf, flower, and root development in Arabidopsis.\(^{8–14}\) indicating that GIF1 is required for normal plant organ growth. Surprisingly, a previous study showed that one mutant allele of GIF1 (an3) promotes seed growth.\(^{48}\) In this study, we have sufficient evidence to support that GIF1 is a positive regulator of seed size in Arabidopsis. For example, loss-of-function of GIF1 formed small seeds, while overexpression of GIF1 produced large seeds (Fig. 5a, c). The gif1 mutation completely suppresses the large and heavy seed phenotypes of myc3 myc4 (Fig. 7c, d). By contrast, the gif1 mutation strongly but not entirely suppresses the seed size and weight phenotypes of kix81-1 kix91-1 and ppd1-2 ppd2-cr (Fig. 7c, d), implying that KIX8/9 and PPDP1/2 might have other mechanisms that act independently of GIF1 to control seed development. These genetic analyses also reveal that GIF1 functions in a common pathway with the KIX-PPD-MYC module to control seed size in Arabidopsis. Consistent with this, KIX8, KIX9, PPD1, PPD2, MYC3, MYC4 and GIF1 have overlapped expression patterns during ovule development and possess partially overlapped expression patterns during early seed developmental stages (Fig. 6 and Supplementary Fig. 14).

STERILE APETALA (SAP/SUPPRESSOR OF DA1, SOD3) acts as a part of the E3 ubiquitin ligase complex to control organ size by regulating the stability of the PPD-KIX complex.\(^{22,23}\) The expression of GIF1 was obviously higher in 35S:SAP plants than that in wild-type plants (Supplementary Fig. 17), indicating that the transcriptional repression of GIF1 is released by the F-box protein SAP that modulates the KIX-PPD complex for 26S proteasome degradation (Fig. 7g). However, SAP did not modulate the stability of MYC3/4 proteins (Supplementary Fig. 19). Without the KIX-PPD complex, MYC3/4 could bind to the promoter of GIF1, but the binding ability is significantly decreased (Fig. 4d). Genetic analyses showed that the gif1 mutation strongly suppresses the large and heavy seed phenotypes of 35S:SAP (Fig. 7c, d), suggesting that SAP and GIF1 act in
a common pathway to regulate seed growth. Based on these genetic and biochemical analyses, we build up a genetic and molecular framework for the SAP-KIX-PPD-MYC-GIF1 module-mediated control of seed size and weight in Arabidopsis (Fig. 7f, g).

Seed size is one of the important targets for plant breeding. In this study, we found that the KIX-PPD-MYC-GIF1 pathway is crucial for seed size control in Arabidopsis. Interestingly, loss-of-function of PPD orthologs in legume *Medicago truncatula* and legume soybean increases seed size and weight as well as leaf size⁴⁰. In pea, mutations in the PPD ortholog ELEPHANT-EAR-LIKE LEAF 1 or the KIX ortholog BIGGER ORGANs cause large flowers and leaves⁴¹. In rice, overexpression of OsGIF1 results in large grains, leaves, and stems, while suppression of OsGIF1 leads to small grains and organs⁴⁰. These findings suggest that the KIX-PPD-MYC-GIF1 pathway may possess a conserved function in different plant species. Thus, it will be interesting to investigate the roles of the KIX-PPD-MYC-GIF1 pathway in crops and utilise their homologues to improve seed size in key crops.

**Methods**

**Plant material and growth conditions.** All of the mutants and transgenic plants were in *Arabidopsis thaliana* Col-0 ecotype. The seeds of ppd1-2 (SALK_057237), ppd2-1 (SALK_142698), kix8-1 (GABI_422H04), kix9-1 (SAIL_1168_G09), gif1 (SALK_150407), myc3 (GK_445B11) and myc4 (GK_491E10) were obtained from the ABRC and NASC, and identified by PCR with T-DNA specific and flanking primers (Supplementary Table 1). kix8-1 kix9-1 plants were obtained by crossing kix8-1 with kix9-1 plants and identified by PCR with primers of kix8-1F/1R and kix9-1F/1R (Supplementary Table 1). gif1 kix8-1 kix9-1 plants were obtained by crossing kix8-1 kix9-1 with gif1 plants and identified by PCR with primers of kix8-1F/1R, kix9-1F/1R and gif1-F/R (Supplementary Table 1). myc3 myc4 plants were obtained by crossing myc3 with myc4 plants and identified by PCR with primers of myc3-F/R and myc4-F/R (Supplementary Table 1). gif1 myc3 myc4 plants were obtained by crossing myc3 myc4 with gif1 plants and identified by PCR with primers of myc3-F/R, myc4-F/R and gif1-F/R (Supplementary Table 1). 3SSSAP plants were described before⁴².

The ppd1-2 ppd2-2 and ppd1-2 ppd2-2 mutants were obtained by CRISPR-Cas9 mediated genome editing⁴². ppd2-2GRNA and ppd1-2GRNA was cloned into the pBluescript-Au6-SK vector, and then Au6-GRNA sequence was transferred to the pCAMBIA1300-pYAOCas9 vector to generate pCAMBIA1300-pYAO-Cas9-Au6-GRNA constructs. The final constructs were transferred into ppd1-2 or ppd2-1 plants by agrobacterium tumefaciens-mediated transformation⁴³. Transgenic plants were screened out with 30 μg ml⁻¹ hygromycin. Genome-edited ppd1-2 ppd2-2 and ppd1-2 ppd2-2 mutants were identified by the sequencing of the PCR products with PPD1 and PPD2 specific primers (PPD1-CRJ1D-F/R and PPD2-CRJ1D-F/R) (Supplementary Fig. 1, Supplementary Table 1). gif1 ppd1-2 ppd2-2 plants were obtained by crossing ppd1-2 ppd2-2 plants with gif1 plants. The gif1 and ppd1-2 ppd2-2 transgenic plants were identified by PCR with primers of gif1-F/R and ppd1-2 ppd2-2F/R. The ppd1-2 mutation in gif1 ppd1-2 ppd2-2 plants was identified by the sequencing of the PCR products with PPD1 specific primers (PPD1-CRJ1D-F/R) (Supplementary Table 1).

The CD8 of GIF1 was obtained from the total RNA of the Col-0 plants with FastQuant RT Super Mix kit (TIANGEN, KR0860). The Neu1-CD8 constructs were transfected into the Kpn1 and Eco1 sites of pMDC32 vector to generate pMDC32-CD8 constructs (Genera, CR0680). The CD8 of MYC3 and MYC4 were obtained from the total RNA of the Col-0 plants with FastQuant RT Super Mix kit (TIANGEN, KR018) and cloned into the Sac I and Kpn1 sites of pMDC32-CFP construct to generate pMDC32-MYC3-MYC4-CFP constructs with EZFusion kit (Genera, GR0686). The CD8s of PPD1, PPD2, and DEL1 were obtained from the total RNA of the Col-0 plants with FastQuant RT Super Mix kit (TIANGEN, KR018) and cloned into the Kpn1 and Eco1 sites of pMDC32-CD8 constructs with EZFusion kit (Genera, GR0686). Constructs were transfected into the *N. benthamiana* leaves by agrobacterium tumefaciens-mediated transformation. The luciferase activity was detected 1 day later after infiltration. One millimolar luciferin (Sigma, 11626353001) was sprayed onto leaves, and the materials were kept in dark for 5 min. Images were obtained with CCD imaging apparatus (CHEMIPROFIT 13080/LND, Roper Scientific).

**FRET-FLIM analysis.** The CFP and YFP sequences were cloned into the Sac I site of pMDC32 vector to generate pMDC32-CFP/YFP constructs with EZFusion kit (Genera, GR0680). The CD8s of MYC3 and MYC4 were obtained from the total RNA of the Col-0 plants with FastQuant RT Super Mix kit (TIANGEN, KR018) and cloned into the Sac I and Kpn1 sites of pMDC32-CFP construct to generate pMDC32-MYC3-MYC4-CFP constructs with EZFusion kit (Genera, GR0686). The CD8s of PPD1, PPD2, and DEL1 were obtained from the total RNA of the Col-0 plants with FastQuant RT Super Mix kit (TIANGEN, KR018) and cloned into the Kpn1 and Eco1 sites of pMDC32-CD8 constructs with EZFusion kit (Genera, GR0686). Constructs were transfected into the *N. benthamiana* leaves by agrobacterium tumefaciens-mediated transformation. The luciferase activity was detected 2 days later after infiltration. One millimolar luciferin (Sigma, 11626353001) was sprayed onto leaves, and the materials were kept in dark for 5 min. Images were obtained with CCD imaging apparatus (CHEMIPROFIT 13080/LND, Roper Scientific).
vectors with EZFusion kit (Genera, GR6086), respectively. Constructs were transferred into GV3101 agrobacterium cells. Agrobacterium cells were grown in LB medium containing 1% (v/v) Trypton, 0.5% (v/v) Yeast Extract, and 1% (v/v) NaCl (pH 7) at 28 °C to 0.8 OD600 concentration. Bacteria were pelleted and resuspended to 0.5 OD600 concentration with the solution containing 10 mM MES (pH 7.5), 10 mM MgCl2, and 150 mM Acetosyringone. The combinations of GFP, MYC3, Myc, PPD2, cYFP, MYC4, pMDC43, and MBP were transformed into GV3101 agrobacterium cells. The YFP fluorescence was observed with the confocal microscope (LSM710, Zeiss, Germany) 2 days later after infiltration. 4′,6-diamidino-2-phenylindole (DAPI, Sigma, D9542) with 2 µg/ml was used to stain the nuclei.

**Pull-down assays.** The CDSs of MYC3 and MYC4 were obtained from the total RNA of the Col-0 plants with FastQuant RT Super Mix kit (TIANGEN, KR108) and cloned into the EcoRI site of pEGX171-GST vector to generate GST-MYC3/4 constructs with EIZusion kit (Genera, GR6086). The CDSs of PPDX and PPDP were obtained from the total RNA of the Col-0 plants with FastQuant RT Super Mix kit (TIANGEN, KR108) and cloned into the BamH I site of pMALC2-MBP vector to generate MBP-PPDX/PPDP constructs with EZFusion kit (Genera, GR6086). The CDSs of KIX8 and KIX9 were obtained from the total RNA of the Col-0 plants with FastQuant RT Super Mix kit (TIANGEN, KR108) and cloned into the EcoRI site of pET24a-His vector to generate His-KIX8/9 constructs with EZFusion kit (Genera, GR6086). Pull-down assays were carried out as a previous study. Constructs were transferred into E. coli BL21 (DE3) cells. All proteins were expressed in E. coli BL21 (DE3) with 0.5 mM Isopropyl β-β-1-Thiogalactoside (IPTG) at 28 °C for 4 h. BL21 (DE3) cells were pelleted and resuspended with the solution containing 50 mM HEPES (pH 7.5), 150 mM NaCl, 1 mM EGTA, 1% (v/v) Triton X-100, 10% (v/v) glycerol, and 1 mM PMSF. Proteins were obtained from bacteria after sonication for 5 min (5 s on, 10 s stop) at 20 amplitude. The combinations of GST-MYC3/4, MBP-PPDX/PPDP, and His-KIX8/9 proteins were incubated at 4 °C for 1 h and pull-down assay was performed on 20% (m/v) bacterial agrose gel. The protein suspension was then loaded onto 15% SDS-PAGE gel and the analysis (Supplementary Fig. 12). Chromatin immunoprecipitation analyses (ChIP-qPCR) were carried out as described previously. Silices were cross-linked with the buffer (0.15 M sucrose, 15 mM PIPES (pH 6.8), 1 mM EDTA, 1 mM PMSF, 1% (v/v) formaldehyde) and vacuumized for 15 min at room temperature. The cross-linking was stop by adding 2 M glycine to final concentration of 100 mM. Silices were washed three times in sterile deionized water and ground to a fine powder with liquid nitrogen. Nuclei were isolation with the buffer (0.25 M sucrose, 15 mM PIPES (pH 6.8), 5 mM MgCl2, 60 mM KCl, 15 mM NaCl, 1 mM CaCl2, 1% (v/v) Triton X-100, 2.5% (v/v) Ficoll 400, 3.12 µl/ml mercaptoethanol, 1x protease inhibitor (cocktail)). Chromatin was extracted with cold nuclei buffer containing 50 mM Tris-HCl (pH 8), 10 mM EDTA, 1% (v/v) SDS after centrifugation at 4°C x 3,000 g for 20 min, and sonicated five times for 10 s at power 6. Chromatin were incubated with ChIP anti-anti body (1:100, Invitrogen, MA3-12526) at 4°C overnight. The coupled-chromatin fragments were pulled down by ChIP protein A + G magnetic beads (1:50, Magna, 16-663) at 4°C for 4 h. The beads were washed for 5 min each time at 4°C with 1 ml of each of the following buffers: 2 times with low salt wash buffer (150 mM NaCl, 20 mM Tris–HCl (pH 8), 0.2% (m/v) SDS, 0.5% (v/v) Triton X-100, and 2 mM EDTA), two times with high salt wash buffer (150 mM NaCl, 20 mM Tris–HCl (pH 8), 0.2% (m/v) SDS, 0.5% (v/v) Triton X-100, and 2 mM EDTA), two times with LiCl wash buffer (0.25 M LiCl, 1% (v/v) sodium deoxycholate, 10 mM Tris–HCl (pH 8), 1% (v/v) NP-40, and 1 mM EDTA) and two times with TE buffer (1 mM EDTA and 10 mM Tris–HCl (pH 8)). DNA was extracted from the beads with elution buffer containing 25% (v/v) SDS, 0.1 M NaHCO3 at 65 °C for 15 min and reversely cross-linked with 100 µl NaCl at 65 °C for 15 min. The DNA was recovered using 60% (v/v) acetic acid and 15% ethanol and rinsed with 100% ethanol to be completely dry. The acetic acid solution was removed with 100 µl of TE buffer, and the final DNA was dissolved in 20 µl of TE buffer. 2 µg DNA was incubated with 1 unit of mungo7 endonuclease (25:24:1). DNA was precipitated with 25% volume of ethanol, 1/10 volume of 3 M sodium acetate (pH 5.2), and dissolved TE buffer containing 1 mM EDTA and 10 mM Tris–HCl (pH 8). qPCR analysis was used to detect the enrichment of the chromatin fragments. 35S:GFP plants were used as the control. The promoter of ACTIN7 was used as a negative control.

**Eelectrophoretic mobility shift assay.** Electrophoretic mobility shift assay (EMSA) was performed according to the procedure of EMSA kit (Thermo). The CDSs of MYC3 and MYC4 were cloned into the pMALC2-MBP vector to generate MBP-MYC3 and MBP-MYC4 constructs, respectively. Proteins were expressed in E. coli BL21 (DE3) with 0.5 mM Isopropyl β-β-1-Thiogalactoside (IPTG) at 28 °C for 4 h. BL21 (DE3) cells were pelleted at 4°C x 5,000 g for 15 min and resuspended with the solution containing 50 mM HEPES (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1% (v/v) Triton X-100, 10% (v/v) glycerol, and 1 mM PMSF. Proteins were purified with anti-MBP agarose beads (NEB, E8037). The interactions were detected with an anti-biotin antibody (1:3,000, Invitrogen, O3-3720).

**Reporting Summary.** Further information on research design is available in the Nature Research Reporting Summary linked to this article.
Data availability
The RNA-seq data are available from the NCBI Sequence Read Archive under accession code PRJNA515854 [http://www.ncbi.nlm.nih.gov/bioproject/615084]. The source data underlying Figs. 1c–g, i–l, 3c–g, i–l, 4a, b, d, 3c–g, i–l, 7c–e, Supplementary Figs. 3a–d, 9a, b, 10a, b, 12 and 15b are provided in a Source Data file.

Received: 2 May 2020; Accepted: 12 March 2020; Published online: 15 April 2020

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Acknowledgements
We thank professor Qi Xie for the system of CRISPR-Cas9 mediated genome editing. We thank the Arabidopsis Stock centres ABRC and NASC for ppd1-2 (SALK_057237), ppd2-1 (SALK_142698), kix8-1 (GABI_422H04), kix9-1 (SAIL_1168_G09), gif1 (SALK_150407), myc3 (GK_445B11), and myc4 (GK_491E10) mutants. This work was supported by the grants from National Natural Science Foundation of China (31872663, 31425004, 3181101602; 91735302; 91535203), the National Special Project (2016ZX08009003-003) and the strategic priority research program of the Chinese Academy of Sciences (XDB27010102).

Author contributions
Y.L. and Z.L. conceived and designed the experiments. Z.L. performed most of the experiments. Y.Z. identified the gif1 mutant. Z.L., N.L. and Y.L. analysed data. Z.L., N.L. and Y.L. wrote the article.

Competing interests
The authors declare no competing interests.

Additional information
Supplementary information is available for this paper at https://doi.org/10.1038/s41467-020-15603-3.

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