The widespread agricultural problem of pre-harvest sprouting (PHS) could potentially be overcome by improving seed dormancy. Here, we report that miR156, an important grain yield regulator, also controls seed dormancy in rice. We found that mutations in one MIR156 subfamily enhance seed dormancy and suppress PHS with negligible effects on shoot architecture and grain size, whereas mutations in another MIR156 subfamily modify shoot architecture and increase grain size but have minimal effects on seed dormancy. Mechanistically, mir156 mutations enhance seed dormancy by suppressing the gibberellin (GA) pathway through de-repression of the miR156 target gene Ideal Plant Architecture 1 (IPA1), which directly regulates multiple genes in the GA pathway. These results provide an effective method to suppress PHS without compromising productivity, and will facilitate breeding elite crop varieties with ideal plant architectures.
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1. a C-to-A single nucleotide polymorphism (SNP) within the reported to affect seed germination in the status of seed dormancy3. Although major progress at phytohormone interaction between these two phytohormones is critical in determining PROTEIN-LIKE targets a group of miR156 target gene, encodes SPL14 and was recently described as mechanisms underlying seed dormancy is far from complete, physiological level has been made, our knowledge of the molecular involvement in seed dormancy is.”

2. In crops, the microRNA miR156 regulates grain yield by modulating plant shoot architecture and grain size2–13. miR156 targets a group of SQUAMOSA-PROMOTER BINDING PROTEIN-LIKE (SPL) transcription factor genes. In rice, the quantitative trait locus (QTL) Ideal Plant Architecture 1 (IPA1), a miR156 target gene, encodes SPL14 and was recently described as a “green revolution” gene for grain yield improvement14. In IPA1, a C-to-A single nucleotide polymorphism (SNP) within the coding region was reported to relieve its repression by miR156, and thus confers an ideal plant architecture with fewer tillers, stronger culms, more grains per panicle, and larger grains7. miR156 also regulates grain size through controlling the expression of SPL16 and SPL1310–12. With expanding knowledge about miR156, the miR156/SPL module has been proposed to be a versatile toolbox to enhance agronomic traits15. However, a comprehensive study regarding the potential functional differentiation of the many genes in the MIR156 family is still lacking, and the molecular mechanisms underlying the various functions of miR156 remain to be revealed.

3. Although miR156 overexpression and knockdown were reported to affect seed germination in Arabidopsis and rice16,17, it is unclear whether miR156 is indeed involved in seed dormancy regulation. Here, through gene mutagenesis by CRISPR/Cas9 in rice, we found that mutations in a MIR156 subfamily (MIR156a–MIR156c, MIR156k, and MIR156l) markedly enhance seed dormancy and suppress PHS, with negligible effects on shoot architecture and grain size. In contrast, mutations in other MIR156 genes (MIR156d–MIR156i) modify plant architecture and increase grain size, but have minimal effects on seed dormancy. We show that miR156 mutations enhance seed dormancy by suppressing the GA pathway through up-regulation of IPA1. Our research reveals in vivo associations of IPA1 with the promoters of many GA biosynthetic, signaling, and deactivated genes, suggesting that IPA1 mediates the effects of mir156 mutations by directly regulating multiple genes in the GA pathway. These results provide an effective method to suppress PHS without compromising productivity, and will facilitate breeding elite crop varieties with ideal plant architectures.

4. **Results**

5. **Group 1 MIR156 genes control shoot architecture.** The rice genome contains 11 MIR156 genes expressing twelve miR156 precursors (pre-miR156a to pre-miR156l), with pre-miR156h and pre-miR156g transcribed from the same gene18 (designated MIR156h in this study). To knockout the eleven MIR156s, we constructed six CRISPR/Cas9 vectors (I–VI) that specifically target the genomic sites corresponding to the mature miR156s (Supplementary Fig. 1a, c). Vector I targets six neighboring genes (group I MIR156 genes: MIR156d–MIR156l) on the phylogenetic tree, and vector II targets MIR156f and MIR156g (Supplementary Figs. 1a and 2). We used an Agrobacterium-mediated method to transform the vectors into Nipponbare, a japonica variety widely used in laboratories, and Xiushui 134 (XS134), an elite japonica cultivar widely cultivated by farmers in southeast China. Using vectors I and II, we obtained many mutant lines of mir156fg, mir156dehi, mir156deghi, and mir156defghi (Supplementary Data 1a, b).

6. Phenoity of these mutants was conducted in paddy fields of Shanghai (China) and Hangzhou (China). During the entire seedling stage, mir156fg and mir156dehi were similar in size to the wild type (Supplementary Fig. 3a–c). mir156deghi and mir156defghi also showed similar size to the wild type before two or three-leaf seedling stage (Supplementary Fig. 3a), but subsequently the seedlings of these two mutants exhibited slightly smaller statures than the wild type (Fig. 1a and Supplementary Figs. 3b, c and 4a). In addition, the leaf blades of mir156dehi, mir156deghi, and mir156defghi seedlings in Nipponbare background were often more erect than those of the wild type (Fig. 1a).

7. At the mature stage, mir156dehi, mir156deghi, and mir156defghi showed apparent changes in plant architecture with significantly fewer tillers when compared with the wild type (Fig. 1b, c and Supplementary Fig. 4b, c). From mir156dehi to mir156defghi, higher-order mutants had fewer tillers (Fig. 1c). In addition, at the mature stage, mir156dehi, mir156deghi, and mir156defghi were taller than the wild type and displayed increased culm diameters (Fig. 1d, e).

8. In Nipponbare background, mir156fg often showed very slightly fewer tillers than the wild type (Fig. 1c and Supplementary Table 1). In XS134 background, obvious differences in tiller number were not observed between mir156fg and the wild type in successive three planting years (Supplementary Fig. 4c). Besides tiller number, we did not observe obvious differences in shoot architecture between mir156fg and the wild type (Fig. 1b–e). Consistent with these observations, we did not detect obvious differences in miR156 abundance and the expression of miR156 target genes between wild-type and mir156fg seedling shoots (Supplementary Fig. 5a–I).

9. To obtain additional group I mutants, we crossed mir156dehi with mir156fg, and identified new combinatorial mutants including mir156deghi, mir156deghi, mir156deghi, mir156deghi, mir156deghi, mir156deghi, mir156deghi, mir156deghi, mir156deghi, and mir156deghi from the segregating F2 and F3 populations in Nipponbare background (Supplementary Data 1a). Overall, among these group I mutants, higher-order mutants showed taller and stronger statures but fewer tillers (Supplementary Table 1).

10. Together, these results indicate that group I MIR156 genes control shoot architecture in rice.
Group II MIR156s have negligible effects on shoot architecture.

Using vectors III–VI (Supplementary Fig. 1a), we obtained many mutant lines of mir156a, mir156b, mir156c, mir156ac, mir156bc, mir156abc, mir156kl, mir156k, and mir156l (Supplementary Data 1a, b). No obvious differences in shoot architecture were observed between these mutants and the wild type in both Nipponbare and XS134 background.

To further study these five MIR156 genes (MIR156a–MIR156c, MIR156k, and MIR156l), a multiplex gene-editing vector (vector VII) was constructed (Supplementary Fig. 1b) and transformed into Nipponbare and XS134 through an Agrobacterium-mediated method. Using this vector, we obtained five independent lines of mir156abckl in each background (XS134 and Nipponbare) (Supplementary Data 1a, b). All the mir156abckl lines showed smaller shoots than the wild type before two or three-leaf seedling stage (Supplementary Figs. 3d–g and 4d), but after this stage, no obvious morphological differences were observed between the wild type and mir156abckl (Fig. 1f–h and Supplementary Fig. 4e–g). We also carefully examined several other agronomic traits, including seed setting rate and grain number per main panicle, and did not observe obvious differences between mir156abckl and the wild type (P > 0.45) (Supplementary Fig. 6a–d). Consistently, the grain yield was not significantly affected by mir156abckl mutations (P > 0.85) (Supplementary Fig. 6e, f). Thus, MIR156a, MIR156b, MIR156c, MIR156k and MIR156l play minimal roles in regulating shoot architecture.

Consistent with the above results, miR156 expression was markedly decreased in mir156deghi seedlings (25-day-old at about five-leaf stage) but not in mir156abckl seedlings (25-day-old at about seven-leaf stage) (Fig. 1i). In addition, transcriptome analyses of the unelongated culms from 20-day-old seedlings revealed that miR156 target genes were not enormously up-regulated by mir156abckl mutations (ratio < 2), whereas several miR156 target genes (SPL3, SPL13, IPA1, and SPL17) were highly up-regulated by mir156deghi mutations (ratio > 2) (Supplementary Fig. 7a, b; Supplementary Tables 2 and 3). Therefore, it is likely that the regulation of shoot architecture is mediated by miR156 through the miR156 target genes, and the MIR156s play a minor role in this process.
Plant growth is suppressed in decuple \textit{mir156} mutants. We obtained two lines of decuple mutant \textit{mir156abcdefghikl} via particle bombardment to co-transform vectors I–VI into Nipponbare (Supplementary Data 1a). Through the crossing between \textit{mir156abckl} and \textit{mir156defghi}, we also identified another \textit{mir156abcdefghikl} line in Nipponbare background (Supplementary Data 1a). These three \textit{mir156abcdefghikl} lines were similarly smaller than the wild type before the heading stage (Supplementary Fig. 8a–d), and showed severely retarded growth during the seedling stage (Fig. 1j–l and Supplementary Fig. 8b). However, at the mature stage, \textit{mir156abcdefghikl} plants were taller than the wild type (Fig. 1m and Supplementary Fig. 8e). In the paddy field, \textit{mir156abcdefghikl} tiller number was slightly larger than that of \textit{mir156defghi}, but smaller than that of \textit{mir156dehi} (Fig. 1n).

We also attempted to obtain mir156 knockout mutants (\textit{mir156abcdefghikl}) by crossing \textit{mir156abcdefghikl} with \textit{mir156defghi}, and two heterozygous \textit{mir156abcdefghikl} plants, in which only \textit{MIR156k} was heterozygous (+/−), were identified in F4 generation. In F5 and F6 generations, most offspring of the heterozygous \textit{mir156abcdefghikl} died during the seedling stage due to severely retarded growth (more severely than \textit{mir156abcdefghikl} seedlings), and the remaining plants were homozygous \textit{mir156abcdefghil} or heterozygous \textit{mir156abcdefghikl}. Among the dead seedlings, we identified homozygous \textit{mir156abcdefghikl} plants (mir156 knockout plants), suggesting that mir156 is essential for survival in rice. Compared with the wild type and \textit{mir156defghi}, the homozygous \textit{mir156abcdefghikl} showed dwarf stature with significantly fewer tillers (Supplementary Fig. 9a, b). Thus, group I and II \textit{MIR156s} act redundantly to support plant growth, especially at the seedling stage.

**Group I \textit{MIR156s} regulate grain size.** Three targets of \textit{miR156} (IPA1, SPL16, and SPL13) positively regulate grain size.\(^{7,10–12}\). Therefore, we examined the grain size and shape of the \textit{mir156} mutants. We found that the grain lengths and 1000-grain weights of \textit{mir156fg}, \textit{mir156dehi}, \textit{mir156defghi}, and \textit{mir156abcdefghikl} were significantly greater than those of the wild type (Fig. 2a, b, e and Supplementary Fig. 4h, i, l). No significant differences in grain width and thickness were observed between the wild type and all \textit{mir156} mutants (Fig. 2c, d and Supplementary Fig. 4j, k). The grain size and shape of \textit{mir156abckl} were similar with those of the wild type (Fig. 2b–e and Supplementary Fig. 4i–l). Moreover, \textit{mir156abcdefghikl} did not show larger grain size than \textit{mir156defghi} (Fig. 2b–e).

These results indicate that group I \textit{mir156} mutations increase grain size, whereas the group II genes play negligible roles in modulating grain size and shape. In addition, the above results also suggest that \textit{mir156fg} is a valuable genetic resource for grain yield improvement. \textit{mir156fg} mutations significantly increased grain size (Fig. 2a, b, e and Supplementary Fig. 4h, i, l) but had minimal effects on shoot architecture (Fig. 1b–e). Besides grain size and shoot architecture, other agronomic traits were not obviously affected by \textit{mir156fg} mutations.

**\textit{mir156} mutations suppress adventitious root formation.** In Arabidopsis, tobacco, and \textit{Malus xiaojinensis}, \textit{miR156} is required for adventitious or lateral root formation.\(^{19,20}\). Therefore, the root development in the \textit{mir156} mutants was investigated. We found that in 20-day-old seedlings, only 1–2 adventitious roots were formed in the decuple mutant \textit{mir156abcdefghikl}, whereas over forty adventitious roots appeared in the wild type (Supplementary Fig. 10a, c). \textit{mir156defghi} and \textit{mir156abckl} seedlings also showed fewer adventitious roots than the wild type (Supplementary Fig. 10b–d). Thus, both group I and group II \textit{MIR156s} are required for adventitious root formation.

**Group II \textit{MIR156s} regulate seed dormancy.** At the sowing time every year in paddy fields, we observed obviously slower seed germination in \textit{mir156abc}, \textit{mir156abcl}, \textit{mir156abckl} and \textit{mir156abcdefghikl} than in the wild type. The slow germination was not clearly observed in group I mutants. Germination assays confirmed the slower germination in \textit{mir156abcdefghikl} and group II \textit{mir156} mutants (Fig. 3a, b and Supplementary Fig. 11a, b). Consistent with these results, mir156 expression was obviously decreased in the germinating embryos of \textit{mir156abckl} and \textit{mir156abcdefghikl} (Supplementary Fig. 12a). These observations indicate that \textit{mir156} may negatively regulate seed dormancy in rice.

To test this hypothesis, we conducted seed germination assays using fresh wet seeds (freshly harvested seeds without drying treatment, and termed fresh seeds hereinafter). We found that \textit{mir156abcdefghikl} fresh seeds hardly germinated after culturing for more than 2 weeks (Fig. 3d), and the group II mutants, including \textit{mir156abc}, \textit{mir156abcl}, and \textit{mir156abckl}, showed obviously slower germination than the wild type and \textit{mir156defghi}, with higher-order mutants exhibiting more delayed germination (Fig. 3c, d and Supplementary Fig. 11c, d). In this assay, \textit{mir156defghi} showed only a slightly slower germination than the wild type (Fig. 3c, d). Thus, group II \textit{MIR156s} play more important roles than the group I in regulating seed dormancy. Consistently, expression analyses revealed that several \textit{mir156} target genes (SPL12, SPL13, and IPA1) were more intensely up-regulated by \textit{mir156abckl} mutations than by \textit{mir156defghi} mutations (Supplementary Fig. 12b–k).

**Group II \textit{MIR156s} control PHS and seed longevity.** In crops, PHS frequently results in severe loss of grain yield and quality. Enhanced seed dormancy is expected to prevent PHS. To explore whether \textit{mir156} mutations suppress PHS, we investigated the PHS rates of the wild type and \textit{mir156} mutants in Nipponbare background in the years 2017 and 2018. In southeast China including Hangzhou, the wet weather during harvest time (late September) often induces severe PHS in Nipponbare. In Hangzhou paddy field, we observed significantly lower PHS rates in \textit{mir156abcl} and \textit{mir156abckl} than in \textit{mir156defghi} and wild-type Nipponbare at the normal harvest time (Fig. 3e and Supplementary Fig. 13). \textit{mir156defghi} showed only slightly lower PHS rates than the wild type (Fig. 3e and Supplementary Fig. 13). These results indicate that group II \textit{mir156} mutations markedly suppress PHS.

Enhanced seed dormancy is also expected to extend seed viability. Therefore, we conducted germination assays using seeds stored for different times. We found that \textit{mir156abckl} lines had significantly higher germination rates than the wild type after storage for 14 months (Fig. 3f), indicating that \textit{mir156abckl} mutations are helpful in maintaining seed viability over time.

**ABA does not support the enhanced seed dormancy in \textit{mir156s}.** Because \textit{ABA} positively regulates seed dormancy, we measured ABA level in fresh seeds of the wild type and \textit{mir156} mutants. The results showed that compared to the wild type, \textit{mir156abcdefghikl} contained severely a decreased level of ABA in the fresh seed embryos (Fig. 4a). ABA level was also significantly decreased in the fresh seeds of \textit{mir156abckl} (Fig. 4b). Thus, enhancing seed dormancy by \textit{mir156} mutations is not through increased accumulation of ABA.
ABA receptors are encoded by the Pyrabactin Resistance 1-like (PYL) family genes\(^{21}\). It was previously reported that mutations in a sub-family of rice PYLs led to severe defects in seed dormancy\(^22\). To explore a potential relationship between miR156 and the ABA pathway in regulating seed dormancy, we crossed miR156 with pyl1/4/6-mir156abckl and identified pyl1/4/6-mir156abckl and pyl1/2/4/6-mir156abckl in the segregating F3 populations. Germination assays with fresh seeds showed thatmir156abckl mutations markedly suppressed the seed dormancy defects in pyl1/4/6 and pyl1/2/4/6, and the seed dormancy of pyl1/4/6-mir156abckl was even stronger than that of the wild type (Fig. 4c, d and Supplementary Fig. 14a, b).

The above results suggest that the enhanced seed dormancy in mir156 mutants is not mediated by the ABA pathway.

**mir156 mutations enhance seed dormancy via the GA pathway.**

To uncover the mechanisms underlying the enhanced seed dormancy in mir156 mutants, we analyzed the transcriptomes of mir156abckl and wild-type fresh seed embryos (Supplementary Data 2). A total of 1132 differentially expressed genes (DEGs) (ratio ≥ 2 or ≤ 0.5, and false discovery rate (FDR) < 0.05), including 729 up-regulated and 403 down-regulated genes in mir156abckl compared to the wild type, were identified (Supplementary Data 3). Among the DEGs, two putative GA receptor genes (LOC_Os06g20200 and LOC_Os09g28630), three key GA biosynthetic genes (GNP1, SDI, and KAO)\(^{23}\), and a putative GA oxidase gene (LOC_Os03g42130) were significantly down-regulated in mir156abckl (Supplementary Data 4). Moreover, a GA deactivating DEG gibberellin 2-oxidase 6 (GA2ox6)\(^{24}\) was markedly up-regulated in mir156abckl (Supplementary Data 4). When lowering the threshold for the DEGs to ratio ≥ 1.5 or ≤ 0.75 (FDR < 0.05), we found down-regulation of another three putative GA receptor genes (LOC_Os08g37040, LOC_Os02g35940, and LOC_Os09g28730) and up-regulation of another two GA deactivating genes (GA2ox8\(^{24}\) and ELONGATED UPPERMOST INTERNODE 1 (EU1)\(^{25,26}\)) in mir156abckl (Supplementary Data 4). These results were validated by quantitative real-time RT-PCR (RT-qPCR) (Fig. 5a). In mir156abckl fresh seed embryos, RT-qPCR also showed decreased expression of the GA biosynthetic and receptor genes but increased expression of the GA deactivating genes (Supplementary Fig. 15).

Next, we measured GA levels in the fresh seed embryos. The results revealed that mir156abckl accumulated much lower levels of two bioactive GAs, GA\(_3\), and GA\(_7\), than the wild type (Fig. 5b). We could not detect other bioactive GAs (GA\(_1\) and GA\(_2\)) in the fresh seed embryos of both mir156abckl and the wild type. Furthermore, the sensitivity of seed germination to GAs was tested using fresh seeds. GA\(_3\)-promoted germination was clearly observed in the wild type during the entire observing time, while the effect of GA\(_3\) on the germination of mir156abckl...
seeds was not clearly observed in the first 5 days, and only from
the sixth day of the treatment, the effect of GA3 on the
germination of mir156abckl seeds became evident (Fig. 5c),
indicating less sensitivity of seed germination to GA3 in
mir156abckl than in the wild type.

Altogether, these results support that mir156 mutations
enhance seed dormancy through suppressing GA biosynthesis
and signaling but promoting GA deactivating.

In addition to enhancing seed dormancy, mir156 mutations
also suppress seedling growth. The morphological phenotype of
mir156abcdfghikl seedlings (Fig. 1j and Supplementary Fig. 8b)
closely resembles those of some GA-deficient mutants, such as
GA-insensitive dwarf 1 (gid1)27, GA-insensitive dwarf 2 (gid2)28,
and strong alleles of SLENDER RICE 1 (SLR1) gain-of-function
mutants29. Consistent with this severely retarded seedling growth,
mir156abcdfghikl seedling shoots contained much lower levels of
the bioactive GAs including GA3, GA4, and GA7 than the wild
type (Fig. 5d). In addition, transcriptome analyses (Supplemen-
tary Data 5) revealed that most of the GA biosynthetic and
signaling genes were down-regulated in mir156abcdfghikl
seedling shoots compared to the wild type (Supplementary Fig. 16;
Supplementary Data 6). Among the DEGs (ratio ≥2 or ≤0.5, and
FDR < 0.05) identified in mir156abcdfghikl and wild-type seedling
shoots, 14 putative GA receptor genes and five key GA
biosynthetic genes (CPS1, KAO, KO2, GNP1, and GA20ox4)23
were significantly down-regulated in mir156abcdfghikl, whereas a
GA deactivating gene GA2ox1024 was markedly up-regulated in
mir156abcdfghikl (Supplementary Data 7). Through the

Fig. 3 mir156 mutations enhanced seed dormancy. a, b Seed germination comparison of the wild type, mir156abckl and mir156abcdfghikl. After harvest, the
seeds for this assay were immediately dried in a 42 °C dry oven for 7 days, and then stored in a 20 °C dry cabinet for 2 weeks. Scale bar, 2 cm. c, d Fresh
seed germination comparison of the wild type, mir156abc, mir156abcl, mir156abckl, mir156defgh, and mir156abcdfghikl. Scale bar, 2 cm. e, f PHS rates of wild-
type, mir156abckl and mir156defghi seeds in Hangzhou in the year 2018. f Germination rates of wild-type and mir156abckl seeds after storage for the
indicated times at room condition in Hangzhou. Each bar in the bar charts represents an independent line. Data are presented as means ± SD. P values
(versus the wild type) were calculated with Student’s t-test. ***P < 0.001. NIP, Nipponbare. Source data are provided as a Source Data file.
Overexpression of the miR156 target gene IPA1. In rice, mir156 acts through targeting 11 SPL genes (SPL2–SPL4, SPL7, SPL11–SPL13, IPA1/SPL14, and SPL16–SPL18). Our transcriptome analyses revealed that among the mir156 target genes, IPA1 was expressed most highly in both mir156abcdfghikl and wild-type fresh seed embryos, and especially in mir156abcdfghikl fresh seed embryos, IPA1 expression level was extremely higher than those of other mir156 target genes (Fig. 6a and Supplementary Table 4). This result suggests that mir156abcdfghikl mutations enhance seed dormancy mainly through increasing IPA1 expression. The transcriptome analyses also revealed that among the mir156 target genes, IPA1 was up-regulated most intensely by mir156abcdfghikl mutations in the seedling shoots (Supplementary Table 5; Supplementary Fig. 18), suggesting that IPA1 also mediates the effects of mir156 mutations on seed dormancy and plant growth.

Overexpression of the miR156 target gene IPA1 also resulted in signifi-
cantly enhanced seed dormancy (Supplementary Fig. 6b–g). In rice, IPA1 also binds to the TGGGCC/T motif via interacting with PCF1 and PCF2. Through sequence analyses of 1300-bp promoter regions, we found that most of the identified GA-related DEGs contained multiple GTAC and/or TGGGCC/T motifs. Therefore, we used ChiP-qPCR to explore associations between IPA1 and the promoters of GA-related genes. We identified above. In P_{SSS}IPA1m:3×FLAG transgenic seedling shoots (see Supplementary Fig. 20a for IPA1 expression in the P_{SSS}IPA1m:3×FLAG lines), the ChiP-qPCR revealed in vivo interactions between IPA1 and the promoters of 14 GA-related DEGs (Fig. 7a–e and Supplementary Fig. 20b–j). The 14 DEGs included five putative GA receptor genes (LOC_Os09g28630, LOC_Os06g20200, LOC_Os02g35940, LOC_Os08g37040, and LOC_Os09g28730), five key GA biosynthetic genes (CPS1, KAO, KO2, GNP1, and SD1), three GA deactivating genes (GA2ox6, GA2ox8, and EUI1), and SLR1. Subsequently, EMSA assays confirmed that IPA1 directly bound to the GTAC-containing fragments in the promoters of KAO,
CPS1, GNPI, SD1, SLR1, GA2ox6, and EU11 (Supplementary Fig. 21a–h). These results suggest that IPA1 directly regulates multiple GA biosynthetic, signaling, and deactivating genes. In the ChIP-qPCR assays, we did not detect interactions between IPA1 and the promoters of the other two DEGs, GA20ox4 and GA2ox10. These two DEGs may be directly regulated by SPL proteins encoded by other mir156 target genes, or may be indirectly regulated by IPA1.

Overall, our results strongly support that mir156 mutations enhance seed dormancy and inhibit seedling growth by suppressing the GA pathway, and IPA1 mediates these effects of mir156 mutations largely by directly regulating multiple genes in the GA pathway.

Discussion

In recent years, with expanding knowledge about mir156, the miR156/SPL module has been proposed as a versatile toolbox to enhance agronomic traits. In crop breeding practices, utilization of the QTLs IPA1, GW8 (SPL16) and GLW7 (SPL13), which modify the regulatory circuit of the miR156/SPL module, have greatly improved grain yield through modulating plant architecture and grain size. Although the miR156/SPL module is powerful in regulating plant architecture and grain size, its pleiotropic effects (e.g., on tillering, tiller angle, plant height, grain shape, etc) often become obstacles for its applications. In addition, miR156 knockdown or the QTL ipa1 leads to extreme changes in plant architecture, whereas crop breeders often need to change plant architecture to less extent. Therefore, breeders are often not able to use the miR156/SPL module to adjust plant architecture at will. In this study, through systematic gene editing, we revealed the functional differentiation of MIR156s in detail, and these results will enable breeders to adjust plant architecture and grain size according to the dosage they need and without undesirable pleiotropic effects (through combinatorial gene editing of group I MIR156s).

In crops, PHS frequently leads to severe loss of grain yield and quality. Enhanced seed dormancy would inhibit PHS. However, the genes known to affect seed dormancy without penalty on productivity are limited. Only two seed dormancy-controlling QTL genes have been identified in crops, including Seed dormancy 4 (Sdr4) in rice and Qsd1 in barley. In bread wheat, a mitogen-activated protein kinase 3 (MKK3) gene was recently proposed as a candidate gene for the seed dormancy-controlling QTL Phs1. Utilizing these few QTL genes requires time-consuming efforts in crossing between different varieties, which may introduce undesirable agronomic traits. No other genes have been reported to mainly affect seed dormancy in crops.ABA positively regulates seed dormancy. Although manipulating the genes in the ABA pathway could enhance seed dormancy, this process would also cause suppression in plant growth and thus reduction in productivity.

In this study, we made the exciting observation that disrupting group II MIR156s (MIR156a–MIR156c, MIR156k, and MIR156l)
markedly enhanced seed dormancy and inhibited PHS with negligible effects on shoot architecture and grain size. Our results also showed that mir156abclk mutations helped to maintain seed viability for longer time. Yield tests in paddy fields showed that mir156abclk mutations did not obviously affect grain yield. Therefore, through MIR156 gene mutagenesis by CRISPR/Cas9, we provided an efficient and effective method to inhibit PHS and increase seed longevity without compromising productivity.

In addition, according to our results, through combinatorial gene editing of group I and group II MIR156s, rice breeders should be able to improve plant architecture, grain size, and seed dormancy simultaneously, and thus generate PHS-resistant crops with increased grain productivity. Given the high conservation of mir156 in plants, this strategy may be also applicable to other crops.

Furthermore, our work shows that miR156 regulates seed dormancy and seedling growth through a molecular network composed of IPA1 and many downstream genes in the GA pathway. Considering the similarity between IPA1 and other miR156-targeting SPLs, as well as mis-regulations of these SPL genes in mir156 mutants (Fig. 6a and Supplementary Table 5), it is plausible that miR156 also regulates the GA pathway through other miR156 target genes (SPL2–SPL4, SPL7, SPL11–SPL13, and SPL16–SPL18). Thus, our study should prompt the readers of our work to speculate that miR156 regulates seed dormancy and plant growth through a complex molecular network which composes of SPLs (including IPA1) and many downstream genes in the GA pathway.

ABA is an important stress phytohormone that enhances plant adaptation to abiotic and biotic stresses. In mir156abcdgfhikl and mir156abckl, we observed decreased ABA levels in seeds. The decreased ABA levels may be a response to the impaired GA pathway in mir156 mutants. Although the ABA level was reduced in seeds, stress resistance does not appear to be compromised in mir156 mutants. Deficiency in GA pathway can improve abiotic stress resistance. In mir156 mutants, impaired GA pathway was observed. Consistent with the impaired GA pathway, we found that group I mir156 mutations reduced transpirational water loss rates of detached flag leaf blades and improved drought stress tolerance of the seedlings (Supplementary Figs. 22 and 23). In addition, it was previously reported that mir156 knockdown or IPA1 overexpression conferred resistance against rice blast and...
bacterial blight, two major devastating diseases in rice. Thus, MIR156 knockout through gene editing may improve both agronomic traits and stress resistance simultaneously.

**Methods**

**Plant material construction and cultivation.** Six single sgRNA-expressing vectors (vector 1–vector VI) were constructed for editing the 11 MIR156 genes (Supplementary Fig. 1a). To generate mir156abckl, a multiplex gene-editing vector (vector VII) was constructed, and in this vector, four sgRNAs expression cassettes were arranged in tandem (Supplementary Fig. 1b). The sgRNAs used in this study were designed to specifically target MIR156 genes. Sanger sequencing was performed to identify mir156 mutants from the transgenic plants. Due to the high sequence similarity between mir156 members, the sgRNAs designed for certain mir156 genes may also induce off-target mutations in other MIR156 genes. Therefore, we sequenced both the target sites and potential off-target sites in MIR156 genes to identify the genotype. In addition, to accurately ascertain the genotype and heritability of every line, we sequenced the target genes in at least two generations and selected T-DNA-free plants for seed harvesting.

In P35S:IPA1 and PcoxIPA1, the IPA1-coding sequence was placed under the control of Os18 and 3SS promoters, respectively, in the pCAMBIA1300 backbone. Through Agrobacterium-mediated transformation, more than thirty transgenic plants. The PHS investigations were conducted in paddy field of Hangzhou at the normal harvest time. In every line, all of the seeds from a plant were investigated in the assays, and the sprouting data of every line were obtained from at least three plants. The PHS investigations were finished in 1 day.

**Seedling investigations.** Seeds having just germinated at similar times were transferred to one-half-strength Kimura B liquid media and cultured in greenhouse (26 °C, 80% humidity, and 12 h light/12 h dark). Then the shoot lengths, fresh weights, and adventitious root numbers of the seedlings were investigated at indicated times. For investigation in paddy field, seeds of the wild type and mir156 mutants were sowed in the paddy field. The seeds from the transgenic plants were sowed in Shanghai (China) and Hangzhou (China) in early June.

**PhS investigation.** PhS was investigated in the paddy field of Hangzhou at the normal harvest time. In every line, all of the seeds from a plant were investigated in the assays, and the sprouting data of every line were obtained from at least three plants. The PHS investigations were finished in 1 day.

**RT-qPCR.** The total RNA was extracted using the TRIzol® Reagent (Invitrogen, Cat. no. 15596018). Reverse transcription was performed using the SuperScript® III Reverse Transcriptase (Invitrogen, Cat. no. 18080-044). Real-time PCR analyses were performed using the Bio-Rad CFX96 real-time PCR instrument and EvaGreen (Biotium, Cat. no. 31000). The PCR was conducted with gene-specific primers designed for the target genes, and Ubi-F and Ubi-R for UBQ10 (Supplementary Table 7).

**Transcriptome analyses.** Two-week-old seedlings were used in the transcriptome analyses of seedling shoots. The materials of unelongated culms, seedling shoots and seed embryos were sampled with three biological repeats for the RNA-seq analyses. The raw reads were extracted and RNAprep pure Plant kit (TIANGEN, Cat. no. DP432), and then libraries were constructed using TruSeq Stranded mRNA (Illumina, San Diego, CA, USA) in accordance with the manufacturer’s instructions. Qualities of RNA-sequ lib sequences were assessed by using a Fragment Analyzer (Advanced Analytical, IA, USA), and the resulting libraries were sequenced using Illumina Hiseq X ten. The raw reads were filtered by removing reads containing adapter and low quality reads for subsequent analyses. Clean reads were aligned to the rice reference genome (TIGR release 7) using Hisat2 with default parameters, and resultant files were input to the cufflinks software for comparative assembly of transcripts and generation of fragments per kilobase of exon per million reads mapped (FPKM). Subsequently, gene expression analyses between the wild type and mir156 mutants were executed using the cufflinks-cuffdiff analysis module.

**Phytohormone measurement.** Plant materials were ground into powder in liquid nitrogen, and extracted with methanol/water (8/2) at 4 °C. The extract was centrifuged at 12,000 g under 4 °C for 15 min. The supernatant was collected and evaporated to dryness under nitrogen gas stream, and then reconstituted in methanol/water (3/7). The solution was centrifuged and the supernatant was collected for LC-MS analysis. The LC-MS analysis was conducted with the API6500 Q TRAP LC/MS/MS system, equipped with an ESI Turbo Ion-Spray interface, operating in a positive ion mode and controlled by Analyst 1.6 software (AB Sciesx).

**Protein extraction and western blot.** For measuring SR1 abundance, total proteins were extracted using a protein extraction buffer (50 mM Tris-HCL pH 7.4, 5 mM EDTA, 0.5 mM DTT, 0.5% Nonidet P-40). Protein concentrations were measured using the BCA protein assay kit (Beyotime, Cat. no. P0010).

**Plant material construction and cultivation.** Six single sgRNA-expressing vectors (vector 1–vector VI) were constructed for editing the 11 MIR156 genes (Supplementary Fig. 1a). To generate mir156abckl, a multiplex gene-editing vector (vector VII) was constructed, and in this vector, four sgRNA expression cassettes were arranged in tandem (Supplementary Fig. 1b). The sgRNAs used in this study were designed to specifically target MIR156 genes. Sanger sequencing was performed to identify mir156 mutants from the transgenic plants. Due to the high sequence similarity between mir156 members, the sgRNAs designed for certain mir156 genes may also induce off-target mutations in other MIR156 genes. Therefore, we sequenced both the target sites and potential off-target sites in MIR156 genes to identify the genotype. In addition, to accurately ascertain the genotype and heritability of every line, we sequenced the target genes in at least two generations and selected T-DNA-free plants for seed harvesting.

In P35S:IPA1 and PcoxIPA1, the IPA1-coding sequence was placed under the control of Os18 and 3SS promoters, respectively, in the pCAMBIA1300 backbone. Through Agrobacterium-mediated transformation, more than thirty transgenic plants. The PHS investigations were conducted in paddy field of Hangzhou at the normal harvest time. In every line, all of the seeds from a plant were investigated in the assays, and the sprouting data of every line were obtained from at least three plants. The PHS investigations were finished in 1 day.

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150 mM NaCl, 2% SDS, 1 mM EDTA, 1 mM dithiothreitol, and 1 μM PMSF), and then separated in a 10% SDS-PAGE gel. After the protein sample was transferred from the SDS-PAGE gel to a Hybond Nitrocellulose membrane (Millipore, Ireland), immunodetection of SLR1 was performed with a rabbit anti-SLR1 primary antibody and an anti-IgG-HRP secondary antibody (Amhrt, China). The anti-SLR1 primary antibody was kindly provided by Donglei Yang17. HRP signal was detected using the SuperSignal West Pico PLUS kit (Thermo Scientific, USA). The blots were captured using ChemiDocXRS+ with Image Lab™ software (BIO-RAD).

ChiP-qPCR. A Pp35S::IPA1m:3 × FLAG vector was constructed in the plasmid pCAMBIA1305 backbone and transformed into XS134 calli. Five Pp35S::IPA1m:3 × FLAG seedlings of T0 generation (about 20-day-old) were used in these assays, and the seedlings of these five Pp35S::IPA1m:3 × FLAG plants were pooled together to conduct the ChiP-qPCR assays. The ChiP assays were conducted based on a previous report47. In brief, 4 g of seedling shoots were collected from Pp35S::IPA1m:3 × FLAG and Pp35S::FLAG seedlings grown in greenhouse (26°C, 80% humidity, and 12 h light/12 h dark). The chromatin complexes were isolated, sonicated and then incubated with monoclonal anti-FLAG antibodies (SIGMA). Precipitated chromatin DNA was analyzed using quantitative PCR (AceQ qPCR SYBR Green Master Mix, Vazyme). The primers used in this experiment are listed in Supplementary Table 8.

EMSA. The primers used to amplify GTAC-containing fragments are listed in Supplementary Table 9. GST-tagged IPA1 was purified from Eraschiella coli BL2 (DE3) cells using Glutathione Sepharose™4B columns (GE Healthcare, U.K.). The master mix was prepared using 40 ng of Cy5-labeled GTAC-containing fragment mixed with 2 μg of the purified protein in 20 mM Tris (pH 7.9), 5% (v/v) glycerol, 200 mM MgCl2, 0.1 M DTT, 4% (v/v) BSA, and 0.5% (w/v) salmon sperm DNA. In the competition tests, the mixture was supplemented with increasing amounts (1:10 to 1:20 or 1:10 to 1:80 mass ratio) of unlabeled DNA salmon sperm DNA. The RNA-seq data generated in this study were deposited in the NCBI's Gene Expression Omnibus (GEO) Database under the accession number GSE131243, and the results of the RNA-seq analyses are available in Supplementary Data 2–7.

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Author contributions
C.M., Z.W., and J.-K.Z. conceived and designed the research. C.M. constructed the mir156 mutants, performed the phytohormone measurements, and conducted the phenotypic and transcriptome analyses. Z.W. conducted the Northern blotting, qRT-PCR, ChIP qPCR, EMSA, and western blotting assays. L.Z. provided the P35S:IPA1 lines. C.M. and Z.W. did other assays together. K.H., X.L., and J.Y. provided assistance in this research. C.M., Z.W., and J.-K.Z. analyzed the data and wrote the paper together. H.S. helped revise the paper and provided useful suggestions. J.-K.Z. oversaw the entire study.

Additional information
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