Mutations in MIR396e and MIR396f increase grain size and modulate shoot architecture in rice

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Introduction

The microRNA miR396, together with its targets the Growth-Regulating Factor (GRF) genes, regulates many aspects of plant growth and development. miR396 negatively regulates plant cell proliferation (Debernardi et al., 2014; Liu et al., 2009; Rodriguez et al., 2009), and its overexpression in Arabidopsis, rice and tobacco led to severely retarded growth (Baucher et al., 2013; Gao et al., 2016a; Liu et al., 2009; Rodriguez et al., 2009; Tang et al., 2017). In Arabidopsis thaliana, the leaves show allometric growth with a specific growth polarity along the proximal-distal axis, and miR396 controls this specific growth pattern via preferential accumulation in the distal part of young developing leaves (Rodriguez et al., 2009). A study surveying 75 eudicot species with different leaf growth polarities showed that the expression gradient of miR396 closely correlated with the polarity of leaf growth (Gupta and Nath, 2015), suggesting a conserved role for miR396 in leaf growth.

In rice, miR396 controls grain yield by regulating grain size and panicle architecture (Che et al., 2016; Duan et al., 2016; Gao et al., 2016a; Hu et al., 2015; Li et al., 2016). The rice grain size quantitative trait locus (QTL) GS2/GL2 encodes GRF4 with a TC → AA transition at miR396-targeting site and thus relieves its suppression by miR396, which further activates brassinosteroid (BR) response to increase the grain size (Che et al., 2016; Duan et al., 2016; Hu et al., 2015; Li et al., 2016). In addition, miR396 modulates panicle architecture through GRF6 (Gao et al., 2016a). In rice, knockdown of miR396 greatly increased GRF6 expression, which further activated auxin biosynthesis and response to generate larger panicles with more grains (Gao et al., 2016a). In tomato, decreasing miR396 expression enlarged flower organs and fruits (Cao et al., 2016). These findings suggest that negative regulation of fruit size is a general role for miR396.

Ideal plant architecture is critical for breeding elite crop varieties. In the past twenty years, plant architecture has been studied intensively, and some key regulators, including SD1, OSDWAR4, DEP1 and IPA1, have been identified and used to improve rice productivity (Huang et al., 2009; Jiao et al., 2010; Miura et al., 2010; Sakamoto et al., 2005; Sasaki et al., 2002). Previous studies regarding plant architectures of monocot crops mainly focused on plant height, tillering, tiller angle, panicle architecture and leaf angle. The lengths of leaf blades and sheaths are also important aspects of plant architecture, but studies on this aspect are very limited in monocot crops.

Despite the known importance for miR396 as a key regulator of plant growth, its exact roles in plant growth and the underlying mechanisms are incompletely known. Here, through systematically mutating MIR396 family genes in rice, we found that MIR396e and MIR396f are important regulators in grain size and plant architecture. miR396f mutations resulted in greatly enlarged grains, lengthened leaf blades and sheaths, as well as shortened upper three internodes. Further analyses revealed that miR396f promotes leaf elongation by increasing the level of a gibberellin (GA) precursor, mevalonic acid (MVA), which subsequently promotes GA biosynthesis. In addition, our results suggest that miR396f mutations suppress internode elongation through decreasing CYP96B4 expression. These findings provide candidate gene-editing targets to breed elite rice varieties.

Summary

Grain size and plant architecture are critical factors determining crop productivity. Here, we performed gene editing of the MIR396 gene family in rice and found that MIR396e and MIR396f are two important regulators of grain size and plant architecture. miR396f mutations can increase grain yield by increasing grain size. In addition, miR396f mutations resulted in an altered plant architecture, with lengthened leaves but shortened internodes, especially the uppermost internode. Our research suggests that miR396f mutations promote leaf elongation by increasing the level of a gibberellin (GA) precursor, mevalonic acid, which subsequently promotes GA biosynthesis. However, internode elongation in miR396f mutants appears to be suppressed via reduced CYP96B4 expression but not via the GA pathway. This research provides candidate gene-editing targets to breed elite rice varieties.

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Results

MIR396 gene editing in rice

The rice genome contains eight MIR396 genes (MIR396a to MIR396h) (http://www.mirbase.org). Sequence analyses revealed that MIR396a, MIR396b, MIR396c, MIR396e and MIR396f locate to intergenic regions, whereas MIR396d, MIR396g and MIR396h locate to the exons of GRF3, GRF2 and GRF1, respectively (Figure S1a). To dissect the function of the MIR396 genes in rice, we constructed a multiplex CRISPR/Cas9 vector to simultaneously target MIR396a, MIR396b, MIR396c, MIR396e and MIR396f (Figure 1a; see Figure S1b for the target sites). We also constructed seven single sgRNA-expressing vectors to mutate MIR396a-MIR396d, MIR396g and MIR396h individually, as well as MIR396e and MIR396f together (see Figure S1b for the target sites). The vectors were separately transformed into Xushui 134 (XS134), an elite japonica cultivar widely cultivated by farmers in south-east China. From T1 to T3 generations, we identified 174 homologous mutant lines for mir396s, including mir396a, mir396b, mir396c, mir396d, mir396e, mir396f, mir396g, mir396h, mir396a,b, mir396abef and mir396acef (line A1–A174, Data S1). We also crossed mir396abef with mir396acef and identified 3 mir396abcf plants in F2 generation (line A175–A177, Data S1). In addition, we obtained mir396e and mir396f plants from the F2 generation of a cross between mir396ef and the wild type (line A178–A184, Data S1).

mir396ef mutations increase leaf length but decrease the lengths of upper three internodes

Phenotypic analyses of the mir396 mutants were conducted in the paddy fields of Hangzhou (China) and Hainan Island (China) under natural conditions. In the paddy field, the mutants including mir396a, mir396b, mir396ab, mir396c, mir396d, mir396e, mir396f, mir396g, and non-frame-shift mir396h (mir396h with non-frame-shift mutations in GRF1) showed similar morphological phenotypes to the wild type during the entire life cycle. In contrast, the other mutants including mir396ef, mir396abef, mir396acef and mir396abcecf showed apparent changes in shoot architecture when compared with the wild type (Figure 1b). At the mature stage in the paddy field, the panicles of mir396ef plants were obviously lower than those of the wild type, but its flag leaves were even higher than the wild type (Figure 1b). To explore this phenotypic difference in detail, we measured the lengths of the leaves and internodes at the seed-filling stage. We found that all the leaf blades and sheaths in mir396ef were significantly longer than those in the wild type (Figure 1d–f), whereas no significant differences in the width of leaf blade were observed between mir396ef and the wild type (Figure 1g). Although the leaf length was remarkably increased, the upper three internodes in mir396ef, especially the uppermost internode (first internode), were significantly shorter than those in the wild type (Figure 1c, h), leading to a defect in panicle exsertion from the flag leaf sheath (Figure 1d, i). Thus, mir396ef mutations modulate shoot architecture by increasing the lengths of leaf blades and sheaths but decreasing the internode length. Higher-order mutants, including mir396aeaf, mir396acef, mir396abcecf, showed similar shoot architectures to mir396ef at the mature stage (Figure 1b).

mir396ef also displayed longer leaf blades and sheaths than the wild type during the seedling stage (Figure S2a–c). During this stage, mir396ef showed more robust growth than the wild type (Figure S2a). Higher-order mutants, including mir396aeaf, mir396acef and mir396abcecf, had similar morphological phenotypes to mir396ef during the seedling stage (Figure S2d). Neither mir396e nor mir396f (whose sequences differ from other mir396 members) was detected in mir396ef and mir396abcecf seedling shoots by northern blot assays, consistent with complete disruption of MIR396e and MIR396f (Figure S3).

To further characterize the shortened internodes and lengthened leaves in mir396ef, we conducted histological analyses to compare the cell size between mir396ef and the wild type. Longitudinal sectioning analyses showed severely shortened cells in the uppermost internode of mir396ef (Figure S4a, b). Epidermal cells in the flag leaf blades and sheaths of mir396ef were slightly longer, but not wider, than those of the wild type (Figure S4c–h). Leaf cross sectioning revealed no obvious differences in cell size between mir396ef and the wild type (Figure S4i). These results indicate that cell elongation is slightly promoted in leaves but markedly suppressed in uppermost internode by mir396ef mutations.

Twelve GRF genes (GRF1–GRF12) have been identified in the rice genome, and among these twelve genes, eleven (GRF1–GRF10 and GRF12) are targeted by mir396 (Choi et al., 2004; Kim and Tsukaya, 2015). Transcriptome analyses in the leaves (leaf blades and sheaths from 50-day-old plants) and developing uppermost internodes revealed that several mir396-targeting GRF genes, especially GRF3, were up-regulated by mir396ef mutations (Figure S5a, b). Among the mir396-targeting GRFs, GRF3 showed the highest expression level in both wild-type and mir396ef leaves and developing uppermost internodes, and was up-regulated by mir396ef mutations more intensely than most other GRF genes (Figure S5a, b), suggesting that GRF3 is involved in the changed elongations of leaves and internodes in mir396ef.

mir396ef mutations increase the sizes of grains and panicles

The grains of mir396ef were obviously larger than those of the wild type (Figure 2a), and the grain length, width and thickness were significantly increased by mir396ef mutations (Figure 2c–e). The 1000-grain weight was increased by about 40% by mir396ef mutations (Figure 2f). Both mir396abef and mir396acef seedlings were similar in size to mir396ef seeds (Figure 2f). mir396abcecf seedlings were much larger than those of mir396ef (Figure S6a–e). However, the seed setting rate was much lower for mir396abcecf than for mir396ef and the wild type (Figure S6f). As we did not observe obviously impaired fertility in mir396a, mir396b, mir396c and mir396abef when compared with the wild type, the severely decreased seed setting rate in mir396abcecf suggests that the five MIR396 genes (MIR396a, MIR396b, MIR396c, MIR396d and MIR396f) function redundantly in affecting fertility. Seeds of the single mutants (mir396a to mir396h) and mir396abef showed similar sizes to wild-type seeds. Consistent with the enlarged grain size in mir396ef, scanning electron microscopy revealed that the epidermal cells of mir396ef spikelet hulls were longer and wider than those of the wild type (Figure S7c–e). Cross sectioning also showed that mir396ef spikelet hulls had obviously enlarged cells (Figure S7a, b).

The panicles of mir396ef were obviously longer and larger than the wild type (Figures 1h and 2b). Therefore, we compared the agronomic characteristics of the panicles between mir396ef and the wild type. We found that the primary branch number per main panicle in mir396ef was slightly larger than that in the wild type (Figure 2g), whereas the numbers of spikelets and secondary
branches per main panicle were not significantly different between mir396ef and the wild type (Figure 2h, i). Therefore, the enlarged panicles in mir396ef accommodated enlarged grains rather than more grains. Higher-order mutants, including mir396aef, mir396abef, mir396acef and mir396abcef, had similar panicle morphologies to mir396ef. Although enlarged grains and panicles were observed in mir396ef, tiller number per plant was not significantly affected by mir396ef mutations (Figure 2j).

The above results indicate that MIR396e and MIR396f are the main regulators of rice growth and development among the MIR396 family genes. Next, using real-time RT-PCR, we analysed the expressions of MIR396e and MIR396f in 20-day-old seedlings.

Figure 1 Shoot architecture analyses of the mir396 mutants. (a) Strategy of vector construction for multiplex gene editing of MIR396s. Red arrows, promoters; violet-blue boxes, sgRNA-expressing sequences. (b) Wild-type, mir396ef, mir396aef and mir396abcef plants at the mature stage. (c) Internode comparison between mir396ef and the wild type. Arrowheads, stem nodes. (d) Comparison of the flag leaf blades and sheaths between mir396ef and the wild type. (e) Leaf blade lengths of the wild type and mir396ef at the mature stage. (f) Leaf sheath lengths of the wild type and mir396ef at the mature stage. (g) Flag leaf blade widths of mir396ef and the wild type. (h) Lengths of panicles and internodes of wild-type and mir396ef main tillers. (i) Quantification of the panicle exsertions from the flag leaf sheaths at the mature stage. Main tillers were used for the investigation. 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; **, P < 0.01. Scale bars, 10 cm.
flag leaves, young panicles (about 3 cm), uppermost internodes and developing spikelets, and found that MIR396e and MIR396f were expressed most highly in young panicle and seedling shoot, respectively (Figure S8a, b).

**mir396ef mutations increase rice productivity**

The above results suggest that mir396ef mutants could be a valuable resource for improving rice productivity. Therefore, we conducted paddy field plot yield test to evaluate the productivity of mir396ef. In Hainan, two independent mir396ef lines showed about 14% and 16% increases in grain yield, respectively (Figure 2k). However, we did not observe increased grain yields for mir396ef in Hangzhou (Figure S9a). Further investigation revealed bad seed filling and low fertility in the enclosed part of mir396ef panicles in Hangzhou (Figure S9b), suggesting that the panicle exsertion defect affected the seed filling and fertility in mir396ef.

**mir396ef mutations promote leaf elongation through the GA pathway**

To explore the mechanism underlying the lengthened leaf blades and sheaths in mir396ef, we analysed the transcriptomes of leaves (including leaf blades and sheaths) from 50-day-old plants (Data S2 and S3). One thousand three hundred and eighty-six differentially expressed genes (DEGs) were identified in the leaves of an mir396ef line (line A1) compared to the wild type (ratio ≥2 or ≤0.5, and false discovery rate (FDR) <0.05), including 873 up-regulated and 513 down-regulated genes (Data S4). Among the DEGs, a putative GA oxidase gene (LOC_Os08g44590, putative GA20ox7), two putative GA receptor genes (designated GID1L2 and GID1L3 here) and four GA-deactivating genes (four gibberellin 2-oxidase family genes including GA2ox6, GA2ox7, GA2ox8 and GA2ox9; Lo et al., 2008) were markedly up-regulated in mir396ef (Data S4 and S5). We also compared the expression profiles of all the detected GA biosynthetic, signalling, deactivating and response genes between wild-type and mir396ef (line A1) leaves, and found that most GA-deactivating and response genes were up-regulated by mir396ef mutations (Data S5 and Figure S10). Transcriptome analysis with another independent mir396ef line (line A6) was also conducted, and the GA-related DEGs (ratio ≥2 or ≤0.5, and FDR <0.05), including GID1L2, GID1L3, putative GA20ox7, GA2ox1, GA2ox3, GA2ox7 and GA2ox8, also showed markedly increased expression in line A6 mir396ef leaves compared to the wild type (Data S6 and S7). We further confirmed these findings using real-time RT-PCR (Figure 3a).
Next, we measured GA levels in the leaves of 50-day-old plants through a high-performance liquid chromatography-mass spectrometry (HPLC-MS) method. In contrast to the increased expression of the GA-deactivating GA2ox genes in mir396ef leaves, much higher levels of the bioactive GAs including GA 3, GA4 and GA 7, as well as a bioactive GA precursor GA 24, were observed in mir396ef compared with the wild type ($P < 0.001$; Figure 3b). Thus, we speculated that the increased expression of GA2ox genes in mir396ef leaves may be a response to the increased GA levels. One of the most significant roles of GAs in plants is to promote organ elongation (Rizza et al., 2017). Therefore, the above results suggest that mir396ef mutations promote leaf elongation by increasing GA levels.

To explain the increased GA levels, we first examined the expression of the key genes in GA biosynthetic pathway, including GA20oxs (GA20ox1, GA20ox2, GA20ox3 and GA20ox4), GA3oxs (GA3ox1 and GA3ox2), CPS1, KS1, KO2 and KAO. In GA biosynthetic pathway, GA20oxs and GA3oxs catalyse multiple late steps, whereas CPS1, KS1, KO2 and KAO catalyse four early steps (Sakamoto et al., 2004). The expression of GA3ox1 and GA20ox3 was not detected in the leaves of 50-day-old wild-type and mir396ef plants, and the expression of other key GA biosynthetic genes did not show substantial differences between mir396ef and the wild type in both transcriptome and real-time RT-PCR analyses (Figure 3a, Data S5 and S7). The results suggest that the increased GA levels in mir396ef leaves do not arise from altered expression of GA biosynthetic genes.

Next, the leaf metabolome of 50-day-old mir396ef was compared with the wild type using a HPLC-MS method which could detect 609 metabolites (Data S8). Through this assay, we detected 37 up-regulated and 26 down-regulated metabolites in mir396ef compared to the wild type (fold change (FC) ≥2 or ≤0.5, and variable importance in projection (VIP) ≥1; Data S9). Interestingly, among the 37 up-regulated metabolites, mevalonic acid (MVA) showed the highest increased level in mir396ef mutants, increasing by about 29 000-fold ($\log_{2}\text{FC} = 14.83$; FC, mir396ef/XS134) (Figure 3c). MVA is a precursor of the terpenoids, which constitute a large class of naturally occurring compounds in plants, including GAs and abscisic acid (ABA; Okada, 2011; Ruiz-Sola et al., 2016). Terpene Synthase (TPS) family genes are responsible for the syntheses of many kinds of...
terpenoids (Falara et al., 2011). Consistent with the increased MVA level, the above transcriptome analyses also revealed significantly increased expression of TPSs in mir396ef leaves (Data S4 and S6). Among the DEGs (ratio ≥2 or ≤0.5, and FDR <0.05) identified in the above transcriptome analyses, 14 and 11 TPSs were found to be markedly up-regulated in line A1 and line A6 mir396ef leaves, respectively (Table S1). Real-time RT-PCR confirmed the increased expression of the TPSs in mir396ef leaves (Figure 3d). The up-regulation of TPS genes in mir396ef leaves may be a response to the increased MVA level.

In plants, MVA is converted to isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP), and then, IPP and DMAPP are used as common precursors of all terpenoid compounds including GAs and ABA (Figure S11; Okada, 2011; Ruiz-Sola et al., 2016). Plants also utilize DMAPP as one precursor of cytokinins (CKs) (Figure S11; Ruiz-Sola et al., 2016). Therefore, we also measured the levels of other phytohormones, including ABA and CKs, in the leaves of 50-day-old plants. Only slightly increased levels of ABA, N6-isopentenyladenine (IP) and trans-zeatin (tZ) were observed in mir396ef, and the levels of other CKs including cis-zeatin (cZ) and dihydrozeatin (DZ) were not significantly increased in mir396ef mutant (Figure S12).

The above results suggest that mir396ef mutations promote leaf elongation by markedly increasing the MVA level, which subsequently promotes GA synthesis to activate the GA pathway.

The inhibited internode elongation in mir396ef mutants is correlated with reduced expression of CYP96B4

To investigate the mechanisms underlying the shortened internodes in mir396ef, we performed RNA-seq analysis of the developing uppermost internode. A total of 453 DEGs in mir396ef (line A1) compared to the wild type (ratio ≥2 or ≤0.5, and FDR <0.05) were observed, including 160 up-regulated and 293 down-regulated genes (Data S10). We did not observe any GA biosynthetic, signalling and deactivating genes among these DEGs. GA content measurement in the developing uppermost internodes revealed that mir396ef contained a markedly increased level of a bioactive GA precursor GA20, whereas other detectable GAs, including GA7, GA19 and GA33, did not show big differences in their levels between mir396ef and the wild type (Figure 4a). In the metabolome analyses, which detected 95 compounds with significantly altered levels (FC ≥2 or ≤0.5, and VIP ≥ 1; Data S11), MVA level did not show significant changes in the developing uppermost internodes between mir396ef and the wild type (FC = 1.23; FC, mir396ef/controls 1.5134). Based on these results, the internode elongation defect in mir396ef cannot be explained by any obvious faults in the GA pathway.

In the transcriptome and real-time RT-PCR analyses, we observed that CYP96B4, mutation of which severely suppressed internode elongation (Ramamoorthy et al., 2011; Tamiru et al., 2015; Wang et al., 2016; Zhang et al., 2014), was significantly down-regulated in mir396ef developing uppermost internode (Figure 4b and Data S10). Loss of CYP96B4 was reported to result in down-regulation of the genes involved in carbohydrate and lipid metabolism (Tamiru et al., 2015; Wang et al., 2016). Enrichment analysis of the DEGs identified in the developing uppermost internodes revealed that many genes involved in carbohydrate and lipid metabolism were significantly down-regulated in mir396ef (Figure 4c and Data S10). These results suggest that mir396ef mutations suppress internode elongation via reduced CYP96B4 expression.

Discussion

Mir396e and MIR396f are valuable gene-editing targets to breed elite crop varieties

Here, we found that mir396ef mutations could enlarge rice grains and panicles, and provided evidence that mir396ef mutations could improve productivity. However, compared with the wild type, mir396ef displayed an obviously deteriorated shoot architecture. In mir396ef, the lengths of leaf blades and sheaths were increased, but the upper three internodes, especially the uppermost internode, were shortened, leading to a defect in panicle exsertion from the flag leaf sheath. Under dry weather condition, this panicle exsertion defect may not affect fertility and seed filling very severely. In Hainan, during the rice-growing season, humid weather was seldom encountered, and therefore, mir396ef showed relatively normal fertility and seed filling. However, in Hangzhou, due to the extremely humid weather, the panicle exsertion defect in mir396ef would markedly affect pollination and make the enclosed panicles prone to diseases, thus severely affecting fertility and seed filling. Therefore, this fault shoot architecture will prevent the application of mir396ef mutations in grain yield improvement.

In mir396ef, if the shortened internodes are restored to normal or even longer lengths, the plant architecture will be greatly improved. Several genes are known to regulate internode elongation in rice. ENLARGED UPPERMOST INTERNODE 1 (EUI1) encodes a GA-deactivating enzyme, and eui1 mutant was morphologically normal until its internodes, especially the uppermost internode, elongated drastically during the heading stage (Luo et al., 2006; Zhu et al., 2006). HOX12 regulates EUI1 expression, and knockdown lines of HOX12 showed a similar morphological phenotype to eui1 (Gao et al., 2016b). Thus, introducing eui1 or hox12 mutations into mir396ef may overcome the defect in internode elongation and thus improve mir396ef shoot architecture to increase productivity.

Mir396ef mutations promote leaf elongation through the GA pathway

mir396 negatively regulates leaf growth in Arabidopsis thaliana through its targets GRF genes (Debernardi et al., 2014; Rodriguez et al., 2009), and the mechanism underlying this role remains obscure. In this work, we observed longer leaf blades and sheaths in mir396ef than in the wild type, indicating that mir396 also negatively regulates leaf growth in rice. Increased GA levels in mir396ef leaves suggest that mir396ef mutations promote leaf elongation through activating the GA pathway.

MVA is an early precursor of GAs (Ruiz-Sola et al., 2016). Our results suggest that mir396ef mutations enhance the GA pathway in leaves through increasing the level of MVA. In plants, MVA is converted to IPP and DMAPP, which are the common precursors of all terpenoid including GAs and ABA (Okada, 2011; Ruiz-Sola et al., 2016). The terpenoid compounds play diverse roles in plants, including attracting insects for pollination, defence against phytopathogenic microbes and enhancing abiotic stress tolerance (Kappers et al., 2005; Stoessl et al., 1976; Wu et al., 2006). Some terpenoids can be used as drugs to combat human diseases (Dewick, 2002). These results imply that MIR396s may be involved in plant pollination and stress resistance through controlling terpenoid synthesis, and genetic manipulation of MIR396s might boost the production of terpenoids in medicinal plants.
mir396ef mutations modulate shoot architecture through two different pathways

Our study suggests that mir396ef mutations promote the elongations of leaf blades and sheaths by promoting the GA pathway. However, mir396ef impaired the elongation of the upper three internodes in the stem, especially the uppermost internode. We did not find any obvious defects in the GA pathway that may explain the suppressed internode elongation in mir396ef mutant plants. Mutation of CYP96B4 was reported to suppress internode elongation (Ramamoorthy et al., 2011; Tamiru et al., 2015; Wang et al., 2016; Zhang et al., 2014). In mir396ef developing uppermost internode, the markedly decreased expression of CYP96B4 suggests that mir396ef mutations impair internode elongation through suppressing CYP96B4 expression. Therefore, our results suggest that mir396ef mutations modulate shoot architecture through two different pathways (Figure 5). The reasons for the opposite effects of mir396ef mutations on the internodes and leaves, as well as how miR396 regulates CYP96B4 expression, remain to be revealed in the future.

GRF genes are involved in forming the phenotypic traits caused by mir396ef mutations

Previous reports identified a major grain size controlling QTL GS2/GL2 (GRF4), one mir396 target gene (Che et al., 2016; Duan et al., 2016; Gao et al., 2016a; Hu et al., 2015; Li et al., 2016). The phenotype caused by mir396ef mutations shows some similarities with that induced by the large-grain GS2/GL2 allele (GRF4 gain-of-function allele), such as lengthened leaves, enlarged grains and increased panicle size. These similarities are reasonable partly because GRF4 expression level is increased in mir396ef compared with the wild type (Figure S5a, b). However, we observed shortened upper three internodes in mir396ef, but similar phenotype was not reported in plants with the GRF4 gain-of-function allele. In addition, the up-regulation of GRF4 in...
mir396ef leaves and uppermost internodes is not very strong (Figure S5a, b). These results strongly suggest that other mir396 target genes are also involved in the phenotypic traits we observed in mir396ef. Our transcriptome analyses in leaves and developing uppermost internodes revealed that several mir396-targeting GRF genes, especially GRF3, were up-regulated by mir396ef mutations (Figure S5a, b). Among the mir396-targeting GRFs, GRF3 showed the highest expression level in both wild-type and mir396ef leaves and uppermost internodes, and was up-regulated by mir396ef mutations more intensely than most other GRF genes (Figure S5a, b), suggesting that GRF3 is intensely involved in the changed elongations of leaves and internodes in mir396ef. How GRF3 regulates the elongations of leaves and internodes remains to be revealed in the future.

**Experimental procedures**

**Vector construction and plant cultivation**

A multiplex gene-editing vector was constructed to edit MIR396a, MIR396b, MIR396c, MIR396e and MIR396f, and in this vector, four sgRNA expression cassettes were arranged in tandem (Figure 1a). To mutate MIR396s individually, seven single sgRNA-expressing vectors were also constructed, among which one vector targets MIR396e and MIR396f, and the others are single MIR396 gene-targeting vectors. All these vectors are specific for the MIR396 gene sequences. The vectors were transformed into XS134 through Agrobacterium-mediated transformation. The transgenic plants were grown in paddy fields under natural conditions. Because the sgRNAs designed for certain MIR396 genes may induce off-target mutations in other MIR396 genes, we sequenced both the target sites and potential off-target sites in MIR396 genes to accurately identify the genotypes. The seeds from the transgenic plants were sowed in the Lingshui County of Hainan Province (China) in late December and in Hangzhou (China) in early June.

**Small RNA northern blot analysis**

Small RNA northern blots were performed as previously described (Pall and Hamilton, 2008) with minor modifications. In brief, twenty μg total RNA was separated by running a 15% SDS-PAGE with 7 M urea and then transferred to a Hybond NX membrane (GE, Amersham). After that, a 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC)-mediated chemical cross-linking was carried out as previously described (Pall and Hamilton, 2008). Antisense complementary oligonucleotides for miRNAs and U6 probes were end-labelled ([α-32P] ATP) by T4 polynucleotide kinase (New England Biolabs). The probe sequences are listed in Table S2.

**Epidermal cell observation and sectioning analyses**

For histological sectioning and epidermal cell observation, the materials were first fixed in FAA’s solution (50% ethanol, 5% glacial acetic acid and 5% formaldehyde). For epidermal cell observation, fresh or fixed leaf blades and sheaths were soaked in boiled water for 10 min, and then transferred to 95% ethanol and boiled for about 1 h until being completely faded. Then, the materials were soaked in 85% lactic acid of 96 °C for 8 min. After cooling to room temperature, the epidermal layers were observed under a microscope. For sectioning analyses, after being dehydrated in a graded ethanol series (70%, 80%, 90% and 100%), the fixed samples were embedded in paraffin. The embedded samples were then sliced into 10–15 μm sections for observation. The transverse sections and epidermal cells were imaged with a Leica DM2500 microscope (Leica Microsystems).

**Plot field tests**

Plants of the wild type and mir396ef were grown in Hangzhou and Hainan paddy fields under natural conditions. The area per plot was 90 × 60 cm, and 24 plants were cultivated in each plot with planting density of 15 × 15 cm.

**Real-time RT-PCR**

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 for the target genes (Table S3), and UBQUITIN was used as the reference gene in the real-time RT-PCR.

**Transcriptome analyses**

The materials were sampled with three biological repeats for RNA-sequencing (RNA-seq) analyses. RNAs were extracted with RNAprep pure Plant kit (TIANGEN, Cat. no. DP432), and then, libraries were constructed using TruSeq Stranded mRNA (Illumina, San Diego, CA) in accordance with the manufacturer’s instruction. Qualities of RNA-seq libraries were assessed by using a Fragment Analyzer (Advanced Analytical, IA), and 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 mir396ef 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...
Metabolome analyses

The metabolome analyses were conducted as previously described (Wang et al., 2017). The freeze-dried materials were crushed to powder, and then, 100 mg powders were weighted and extracted overnight at 4°C with 1 mL 70% aqueous methanol. Following centrifugation at 10 000 g for 10 min, the extracts were filtrated (SCAA-104, 0.22 μm pore size; ANPEL) before LC-MS analysis.

The sample extracts were analysed using an LC-ESI-MS/MS system (HPLC, Shim-pack UPLC SHIMADZU CBM30A system; MS, Applied Biosystems 4500 Q TRAP). The analytical conditions were as follows: HPLC: column, Waters ACQUITY UPLC HSS T3 C18 (1.8 μm, 2.1 × 100 mm); solvent system, water (0.04% acetic acid): acetonitrile (0.04% acetic acid); gradient program, 100:0 V/V at 0 min, 5:95 V/V at 11.0 min, 5:95 V/V at 12.0 min, 95:5 V/V at 12.1 min, 95:5 V/V at 15.0 min; flow rate, 0.4 mL/min; temperature, 40°C; and injection volume, 5 μL. The effluent was alternatively connected to an ESI-triple quadrupole-linear ion trap Q TRAP-MS.

LIT and triple quadrupole (QQQ) scans were acquired on a triple quadrupole-linear ion trap mass spectrometer (Q TRAP), API 4500 Q TRAP LC/MS/MS system, equipped with an ESI Turbo ion-Spray interface, operating in a positive ion mode and mass calibration were performed with 10 and 100 μg/mL polypropylene glycol solutions in QQQ and LIT modes, respectively. QQQ scans were acquired as MRM experiments with collision gas (nitrogen) set to 5 psi. DP and CE for individual MRM transitions were done with further DP and CE optimization. A specific set of MRM transitions was monitored for each period according to the metabolites eluted within this period.

Accession numbers

The sequence data of the MIR396 genes can be found in the mirBase database (http://www.mirbase.org/) under the following accession numbers: MIR396a, M10001046; MIR396b, M10001047; MIR396c, M10001048; MIR396d, M10001304; MIR396e, M10001703; MIR396f, M10001563; MIR396g, M10001304; and MIR396h, M10001304. The sequence data of the other genes can be found in the MSU database (http://rice.plantbiology.msu.edu/) under the following gene locus identifiers: GRF1, LOC_Os02g53690; GRF2, LOC_Os06g10310; GRF3, LOC_Os04g51190; GS2/GL2/GRF4, LOC_Os02g47280; GRF5, LOC_Os06g02560; GRF6, LOC_Os03g51970; GRF7, LOC_Os11g29980; GRF8, LOC_Os11g35030; GRF9, LOC_Os03g47140; GRF10, LOC_Os02g45570; GRF12, LOC_Os04g48510; GA2ox1, LOC_Os03g63970; GA2ox2, LOC_Os01g66100; GA2ox3, LOC_Os07g07420; GA2ox4, LOC_Os05g34854; GA3ox1, LOC_Os05g08540; GA3ox2, LOC_Os01g08220; CPS1, LOC_Os02g17780; KSI, LOC_Os04g52230; KAO, LOC_Os06g02190; KO2, LOC_Os06g37364; GID1L2, LOC_Os03g57640; GID1L3, LOC_Os07g34370; GA2ox1, LOC_Os05g06670; GA2ox3, LOC_Os01g55240; GA2ox6, LOC_Os04g44150; GA2ox7, LOC_Os01g11150; GA2ox8, LOC_Os05g48700; GA2ox9, LOC_Os02g41954; TP53, LOC_Os08g04500; TP520, LOC_Os04g27340; TP524, LOC_Os04g27790; CYP96B4, LOC_Os03g04680.

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Conflict of interest

The authors declare no conflict of interest.

Author contributions

C.M. and D.W. conceived and designed the research. C.M. constructed the plant materials, performed the phytohormone measurements and conducted the phenotypic, transcriptome and metabolome analyses. D.W. and R.H. conducted the northern blotting and real-time RT-PCR analyses. C.M. and D.W. did the other assays together. All the authors analysed the data together. C.M. and D.W. wrote the manuscript together. C.M., D.W. and J.-K.Z. oversaw the entire study.

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Supporting information

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

Figure S1. MIR396-GRF gene structures and target sites of MIR396e gene editing.

Figure S2. Comparison of wild-type and mir396ef seedlings.

Figure S3. Detection of miR396e and miR396f in seedling shoots by Northern blotting analyses.

Figure S4. mir396ef mutations increase the cell lengths of leaf blades and sheaths but decrease the cell length of uppermost internode.

Figure S5. Relative expression analyses of miR396 target genes in leaves and developing uppermost internodes.

Figure S6. Seed size and fertility analyses of the wild type, mir396ef and mir396a/bcfe in Hangzhou.

Figure S7. mir396ef mutations enlarged the cells of spikelet hulks.

Figure S8. Real-time RT-PCR analyses of MIR396e and MIR396f expressions.

Figure S9. Grain yield investigation of the wild type and mir396ef in Hangzhou.

Figure S10. Expression profiles of GA biosynthetic, signaling, deactivating and response genes in the leaves of 50-day-old wild-type and mir396ef plants.

Figure S11. Terpenoid biosynthetic pathway (Ruiz-Sola et al., ).

Figure S12. Endogenous ABA and CK levels in the leaves of 50-day-old wild-type and mir396ef plants.

Table S1. The list of rice mir396ef mutants.

Table S2. Primers used in the Northern blot assays.

Table S3. Gene expression profiles in wild-type and mir396ef (line A1) leaves.

Table S4. Gene expression profiles in wild-type and mir396ef (line A6) developing uppermost internodes.

Table S5. Expression profiles of the DEGs identified in wild-type and mir396ef (line A1) leaves.

Table S6. Expression profiles of the DEGs identified in wild-type and mir396ef (line A6) leaves.

Table S7. Expression profiles of the GA-related genes in wild-type and mir396ef (line A6) leaves.

Table S8. The metabolites detectable in the metabolome analyses.
Data S9. The metabolites with significantly different levels between wild-type and mir396ef leaves.

Data S10. Expression profiles of the DEGs identified in wild-type and mir396ef developing uppermost internodes.

Data S11. The metabolites with significantly different levels between wild-type and mir396ef developing uppermost internodes.