Overexpression of miR164b-resistant OsNAC2 improves plant architecture and grain yield in rice

Dagang Jiang1, Weiting Chen1, Jingfang Dong1, Jing Li1, Fen Yang1, Zhichao Wu2, Hai Zhou1, Wensheng Wang2 and Chuxiong Zhuang1,*

1 State Key Laboratory for Conservation and Utilization of Subtropical Agro-bioresources, College of Life Sciences, South China Agricultural University, Guangzhou 510642, China
2 Institute of Crop Sciences, Chinese Academy of Agricultural Sciences, Beijing 100081, China

* Correspondence: zhuangcx@scau.edu.cn

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Abstract

Plant architecture is a major target of rice (Oryza sativa) breeding and selection, breeding and selection, but the underlying regulatory networks remain unclear. Here, we overexpressed an OsNAC2 mutant (OErN) that cannot be cleaved by the miRNA miR164b. OErN plants had better plant architecture and longer panicles, and produced more grains. The parental line averaged 12.2 primary and 31.5 secondary branches in the main panicles; two OErN lines averaged 15.0 and 15.2 primary, and 41.5 and 44.3 secondary branches. In large-scale field trials, OErN plants produced at least 58.62% more total grain (by weight) compared with the parental line. They also had more large and small vascular bundles in the stem internodes and leaves. Overexpression of miR164b or down-regulation of OsNAC2 led to decreased panicle length and grain yield in the main panicle. The OErN plants showed significant up-regulation of the grain number and plant architecture-related genes IPA1 and DEP1. A survey of >3000 rice varieties found no natural mutations in the miR164b-binding site of OsNAC2. OErN increased yield in Nipponbare and the commonly grown Yangyujing 3 cultivars. In summary, we identified an efficient new strategy to increase rice yield substantially and improve plant architecture through overexpression of OsmiR164b-resistant OsNAC2.

Keywords: Architecture, miRNA, OsNAC2, rice (Oryza sativa L.), yield.

Introduction

Increases in population, reductions of available farmland, and environmental deterioration could lead to worldwide food shortages. Therefore, the production of crop varieties with a higher yield per unit area remains of critical importance (McClung, 2014). An estimated 40% increase in rice production will be required by 2030 to support the predicted population growth. Although hybrid rice breeding resulted in a 10–20% increase in grain yield (Khush, 2005), meeting the increasing demand will require new strategies.

Plant architecture encompasses branching pattern, plant height, leaf shape and arrangement, and inflorescence morphology (Reinhardt and Kuhlemeier, 2002; Wang and Li, 2011). Rice breeding strategies for obtaining high-yield varieties have selected for a so-called ideal plant architecture (IPA); these ideotype breeding strategies select for plants that have an optimal combination of low tiller numbers, few unproductive tillers, more grains per panicle (compared with currently cultivated varieties), and thick and sturdy stems (Wang and Li, 2011). Several plant architecture-related genes, corresponding to some yield quantitative loci (QTLs), such as Gn1a, GS3, Ghd7, and Ghd8, have been identified (Ashikari et al., 2005; Song et al., 2007; Xue et al., 2008; Yan et al., 2011).

In rice, the Ideal Plant Architecture 1 gene (IPA1) controls the establishment of plant architecture and increases
yield potential (Jiao et al., 2010; Miura et al., 2010). IPAI encodes SQUAMOSA PROMOTER BINDING PROTEIN-LIKE14 (OsSPL14), and IPAI controls tillering and panicle morphology through the regulation of the downstream gene DENSE AND ERECT PANICLE1 (OsDEP1) (Lu et al., 2013). IPAI is regulated at the transcriptional and post-transcriptional levels. For example, a recent study reported that the presence of three tandem repeats upstream of IPAI, a naturally occurring variant, can enhance grain yield in super rice varieties such as Yongyou12 (YY12) and related varieties (Zhang et al., 2017). IPAI INTERACTING PROTEIN 1 (IPI1), a RING-finger E3 ligase that can interact with IPAI in the nucleus, promotes the degradation of IPAI in panicles but stabilizes IPAI in shoot apices (Wang et al., 2017). In plants with optimal expression of IPAI, breeding and selection can then be used to fine-tune other IPA traits; this may lead to improved yield (Zhang et al., 2017).

Ongoing work has shown that miRNAs participate in the regulation of many plant architecture-related genes (Zheng and Qu, 2015). For example, OsmiR156 regulates IPAI. OsmiR156 also regulates other SPL genes, which control panicle morphology, grain number, and plant architecture (Jiao et al., 2010; Miura et al., 2010; Wang et al., 2012; Si et al., 2016). OsmiR397 influences overall yield by controlling grain number and branches per panicle, via down-regulation of its target gene OsLAC (Zhang et al., 2013). OsmiR396b suppresses the expression of growth-regulating factors (GRFs) and regulates GRF/GIF (GRF-interacting factor)-mediated processes in rice growth and development, including grain size, grain number, panicle morphology, and grain yield (Che et al., 2015; Duan et al., 2015; Gao et al., 2015). A natural mutation in GRAIN SIZE ON CHROMOSOME 2 (GS2) perturbs the binding site for OsmiR396c and results in increased grain size and yield (Hu et al., 2015; Li et al., 2016). OsmiR396d regulates spikelet development through regulation of the expression of a spikelet-specific subfamily of OsGRF genes, and enhancing the expression of OsJMJ706 and OsCR4 (Liu et al., 2014). OsmiR172 regulates the expression of APETALA2 (AP2)-like transcription factor genes to affect branch and tiller numbers (Zhu et al., 2009; Wang et al., 2015). Moreover, overexpression of OsmiR444a reduces the number of tillers through the repression of OsMADS57 (Guo et al., 2013). The OsNAC2 locus, one of the target genes of miR164b, encodes a key transcription factor involved in rice development (Mao et al., 2007; Fang et al., 2014; Chen et al., 2015).

Molecular characterization of genes controlling rice plant architecture and grain yield, and examination of their regulation will inform efforts to improve plant architecture and breed high-yield rice. In the present study, we overexpressed OsNAC2 by mutating the OsmiR164b-binding site sequence. The resulting transgenic plants showed increased grain number and better plant architecture, which resulted in significantly improved grain yield per plant and overall rice yield in paddy fields. By manipulating OsNAC2 expression, our work provides a new and practical approach for rice breeders to increase rice production.

Materials and methods

Plant and other experimental materials

Rice (Oryza sativa L.) plants were grown in a paddy field at the South China Agricultural University under natural conditions. Zhonghua 11 (ZH11), Yangyunjing 3, and Nipponbare were used as the wild type for analyses. At least 10 plants were used to measure agricultural traits for each experiment. Escherichia coli DH10B and Agrobacterium tumefaciens EHA105 were used for cloning and transformation experiments. pCAMBIA 1380 was used as the binary vector for Agrobacterium-mediated transformation.

For observing rice roots, rice seeds were germinated at room temperature. The seedlings were transplanted in Kimura B nutrient solution. The roots were separated one by one before imaging on a scanner at the stage of flowering, and the roots were measured following the previously described method (Zhao et al., 2004).

Vector construction and genetic transformation

For overexpression of the miRNA, the precursor OsmiRNA164b was isolated by PCR using primers Ox164F and Ox164R. The PCR products were cloned into the PstI–HindIII sites of pCAMBIA1380-Ubi containing the maize Ubiquitin promoter to construct the overexpression vector.

To overexpress OsNAC2, the ORF was amplified from cDNA using primers OxnacF and OxnacR. After subcloning and sequencing, the correct gene fragment was inserted into pCAMBIA1380-Ubi (driven by the Ubiquitin promoter).

To overexpress the miRNA164b-resistant OsNAC2, OxnacF and specific primers OxnacRm carrying the 5 bp mutations, OxnacFm carrying the 5 bp mutations, and OxnacR, respectively, were used to amplify two fragments of the OsNAC2 sequence. After purification, the two fragments were mixed in equal molar ratios and used as the PCR template to amplify the full-length ORF of OsNAC2 with the miRNA164b-resistant mutation, using OxnacF and OxnacR. The full-length ORF with the 5 bp mutations was then introduced into pCAMBIA1380-Ubi after sequencing.

To make the RNAi constructs, a fragment of OsNAC2 was isolated by PCR using the primers RNA1 and RNA2 from the rice ZH11 genome into the EcoRI–HindIII sites and from immature panicle cDNA with primers RNA12 and RNA13 into the HindIII–SalI sites of the cloning vector of Bluescript II. The RNAi construct was driven by the native promoter of OsNAC2. The OsNAC2 promoter was amplified from the rice genome using the primers PRNA1F and PRNA1R, and cloned into the BamHI–EcoRI sites of the cloning vector Bluescript II with RNAi fragments. After sequencing, the total fragments containing the native promoter and RNAi fragments were digested with BamHI and SalI, and cloned into the binary vector pCAMBIA1380. The cloning was performed following the previously described method (Li et al., 2011; Zhou et al., 2014). The primer sequences are listed in Supplementary Table S2 at JXB online.

Co-segregation analysis between the transgene and phenotype was performed using plants in the T1 and T2 generations to evaluate the effects of the transgenes.

RNA extraction and quantitative real-time PCR (qRT-PCR)

RNA was isolated using the RNA extraction kit TRizol Reagent (Invitrogen, USA) and quantified with DU730 (Beckman Coulter, Germany). Briefly, ~3 µg of total RNA was used to synthesize the first-strand cDNA. qRT-PCR was performed with the SYBR Premix ExTaq kit (TaKaRa) in a total volume of 20 µl on the BIO-RAD CFX connect following the manufacturer’s manual. Data were normalized to the internal rice ubiquitin (UBI) gene and the relative quantification method was used for data analysis. RT-PCR was performed with TaKaRa Ex Taq Hot Start Version following the manufacturer’s manual.
For quantification of mature OsmiR164b, stem–loop reverse transcription–quantitative PCR was performed as described (Chen et al., 2005; Zhou et al., 2012). All reactions were run in triplicate.

Subcellular localization of OsNAC2

The OsNAC2 cDNA sequence was cloned into the pUC18 vector with the coding sequence for enhanced green fluorescent protein (eGFP) under the control of the Cauliflower mosaic virus (CaMV) 35S promoter provided by Yang et al. (2014). Dehulled rice seeds of ZH11 were surface-sterilized using 1.5% sodium hypochlorite for 30 min, then germinated and cultured on half-strength Murashige and Skoog medium in the tissue culture room for ~7 d. Seedlings were grown hydroponically under natural light for 3 d. The protoplast isolation and transformation were performed following the method of Yang et al. (2014).

Histological sectioning

The stems were collected from wild-type and transgenic plants at the flowering stage and sliced with a sharp blade. The sections were stained in 0.1% aqueous safranin for 3 min and washed in tap water for 1–3 min. The photos were taken with a light microscope.

Measuring brown and milled rice percentages

For measuring the percentage of brown rice and milled rice, harvested paddy rice was completely dried. The process was performed following the previously described method (Tan et al., 2000). The chalkiness rate was determined following the method of Xu and Chen (2016).

Sequence analysis

We analyzed the single nucleotide polymorphisms (SNPs) in the gene Os04g0460600 (including the promoter region) from the 3000 rice accessions of the 3000 genomes project, according to previously described methods (Li et al., 2014; Alexandrov et al., 2015).

Accession numbers

Sequence data from this article can be found at the Rice Genome Annotation Project website or miRBase data libraries under the following accession numbers: An1-1 (LOC_Os04g28280), DST (LOC_Os03g57240), HTD1 (LOC_Os04g46470), HTD2 (LOC_Os03g10620), SP1 (LOC_Os11g12740), Ghd7 (LOC_Os07g15770), DTH1 (LOC_Os08g07740), IP1 (LOC_Os08g39890), DEP1 (LOC_Os09g26999), OsNAC2 (LOC_Os04g38720), and Ubi (LOC_Os03g13170).

Results

OsNAC2 is a target gene of miR164b

We cloned OsNAC2 from a cDNA library of young rice panicles. We analyzed the subcellular localization of OsNAC2 using a transiently expressed GFP fusion in transformed rice protoplasts and found that OsNAC2–GFP localized to the nucleus (Supplementary Fig. S1). To overexpress OsNAC2, we cloned OsNAC2 downstream of the maize Ubil promoter and transformed this construct into the rice variety ZH11 to produce 11 independent transgenic lines (ZH11-Ubil-overexpression OsNAC2, ZUOEN). However, no significant difference in morphology or agronomic traits was observed between the overexpression lines (lines ZUOEN 4 and ZUOEN 6) and wild-type ZH11 (Supplementary Fig. S2; Supplementary Table S1). RT-PCR analysis of the ZUOEN 4 and ZUOEN 6 lines revealed that the transcript level of OsNAC2 was not significantly altered in the transgenic lines (Supplementary Fig. S2D), indicating that the OsNAC2 mRNA levels might be post-transcriptionally regulated.

A previous study demonstrated that OsNAC2 is a target gene of miR164b (Fang et al., 2014) (Supplementary Fig. S3). We further verified that OsNAC2 is a target of miRNA164b by analyzing the sequence through the http://www.mirbase.org/ website. To verify the potential effect of miR164b binding on OsNAC2 transcript levels, we introduced point mutations in the predicted miR164b-binding site without introducing any amino acid changes, and overexpressed this mutated version of OsNAC2 using the Ubi1 promoter (Ubi-overexpression miR164b-resistant OsNAC2, UOErN) in ZH11 (Supplementary Fig. S3). Of the 14 independent transgenic lines we produced (ZH11-UOErN, ZUOErN), 11 displayed obvious phenotypic alterations such as vigorous growth (Fig. 1A) and enlarged main panicles (Fig. 1B, C). RT-PCR analysis showed increased OsNAC2 transcript levels in lines ZUOErN3 and ZUOErN4 (Fig. 1D). These results suggest that increased levels of OsNAC2 cause morphological changes in ZUOErN plants.

Overexpression of miR164b-resistant OsNAC2 increased grain number and yield

The ZUOErN plants showed better plant architecture compared with the wild-type (WT) ZH11 parental line. To test for any potential yield change in ZUOErN3 and ZUOErN4, we performed statistical analyses on single homozygous T3 plants. The grain yields for ZUOErN3 and ZUOErN4 in the early cropping season of 2011 were 22.07 ± 3.16 g and 20.18 ± 3.18 g per plant, respectively. Compared with grain yield of 11.43 ± 2.22 g per plant in ZH11, the production increased by 93.08% and 76.55% in ZUOErN3 and ZUOErN4, respectively (Table 1). Similarly, ZUOErN3 and ZUOErN4 produced 22.97 ± 2.37 g and 21.05 ± 3.81 g of grain per plant in the late cropping season, respectively, an increase of 78.75% and 63.81% compared with ZH11 (12.85 ± 3.28 g per plant) (Table 1). The grain yield per plant was consistently higher in ZUOErN3 and ZUOErN4 under various seasonal conditions, indicating that these lines had stable yields. Furthermore, the ZH11 and the transgenic lines showed no difference in the brown rice or milled rice percentage (Supplementary Fig. S5A, B). Moreover, we observed no obvious difference in grain phenotype among the WT, ZUOErN3, and ZUOErN plants (Supplementary Fig. S5D), although the chalkiness rate was higher in the transgenic lines than in the WT (Supplementary Fig. S5C).

To evaluate the yield of ZUOErN plants in the field, in accordance with the approval document for field trials of genetically modified organisms from the Chinese Ministry of Agriculture (Nongji’an 2011-T013), field breeding and yield measurements were performed during the early cropping season of 2012 (Supplementary Fig. S4). Statistical analysis showed that, when compared with ZH11, the total yield of ZUOErN3 and
ZUOErN4 increased by 63.06% and 58.62% in the field, respectively (Supplementary Fig. S4; Table 1), indicating that transgenic plants displayed increased yield potential under field conditions, which is consistent with the yield increase in single plants.

To investigate the reason for the increased grain number (Fig. 1E, F), we examined the main yield-related factors in the ZUOErN lines. The homozygous T<sub>3</sub> ZUOErN3 and ZUOErN4 plants produced longer main panicles (25.97 ± 1.91 cm and 27.17 ± 1.64 cm, respectively), in comparison with ZH11 plants (22.27 ± 1.16 cm) (Fig. 1B, C). The number of primary branches per main panicle increased from 12.2 ± 1.21 in ZH11 to 15.0 ± 1.23 and 15.2 ± 0.89 in ZUOErN3 and ZUOErN4, respectively (P=5.045 × 10⁻³ and P=9.68 × 10⁻⁴ (Fig. 1G)). Also, the number of secondary branches per main panicle increased by 32.7% and 40.6% in ZUOErN3 (41.75 ± 4.60) and ZUOErN4 (44.33 ± 7.41) plants (P=1.372 × 10⁻² and P=1.422 × 10⁻², respectively) (Fig. 1H). Moreover, the number of grains per main panicle significantly increased (P=3.192 × 10⁻³ and P=2.343 × 10⁻³, respectively) in ZUOErN3 (212.0 ± 25.5) and ZUOErN4 (220.3 ± 23.2) when compared with the values in ZH11 (160.3 ± 22.3) (Fig. 1E, F). These data indicate that the higher..
Manipulating NAC2 expression improves rice architecture and grain yield

grain numbers in the overexpression lines are the result of increased panicle length and increased number of primary and secondary branches per panicle. However, we found no significant differences in 1000 grain weight or seed setting rates in ZUOErN3 and ZUOErN4 plants when compared with ZH11 plants (Supplementary Table S1). These data suggested that the increased yield in overexpression lines results from the increased numbers of grains produced.

Because the overexpression of OsNAC2 caused an overall phenotypic change (Fig. 1A), we next analyzed effective tiller number. ZUOErN3 and ZUOErN4 plants produced an average of 9.93 ± 2.21 and 10.0 ± 2.49 productive tillers (P=3.468 × 10⁻³ and P=8.910 × 10⁻³), respectively, both of which are significantly higher than the productive tiller number of ZH11 plants (6.67 ± 1.11). In addition, the morphology of the tiller panicles resembled that of the main panicles of ZUOErN lines (Supplementary Fig. S6). In summary, ZUOErN plants displayed a moderate increase in productive tiller number, panicle length, branching number per panicle, grain number, and yield.

The effects of OsNAC2 on the stem, flag leaf, and root

ZUOErN plants exhibited more vigorous growth and stronger stems than WT plants (Figs 1A, 2A), so we measured their agronomic traits related to the stem, flag leaf, and root. The stems of ZUOErN were thicker and stronger than those of WT plants (Figs 1A, 2A). Furthermore, the diameter of the first internode was 3.4 ± 0.17 mm in ZH11, whereas the diameter was 3.8 ± 0.19 mm in ZUOErN3 plants. The diameters of the second and third internodes of ZH11 plants were 4.6 ± 0.19 mm and 5.6 ± 0.17 mm, whereas they measured 5.0 ± 0.18 mm and 6.4 ± 0.22 mm, respectively, in ZUOErN3 plants (Fig. 2B, C).

Cross-sections of the stem internodes showed that the number of large vascular bundles in the first internode was significantly higher in ZUOErN3 and ZUOErN4 plants (17.0 ± 0.71 and 17.6 ± 0.89) (P=3.435 × 10⁻⁶ and P=7.583 × 10⁻⁶), when compared with ZH11 plants (12.3 ± 1.41) (Fig. 2E). The number of small vascular bundles was also significantly higher, 24.2 ± 1.58 in ZH11 plants in comparison with 34.2 ± 2.77 and 34.5 ± 2.87 in ZUOErN3 and ZUOErN4 plants (P=1.597 × 10⁻⁶ and P=1.462 × 10⁻⁶) (Fig. 2E). Transection showed that the stem had more cells, but cell size was similar to that of ZH11 (Fig. 2D). In addition, the number of large and small vascular bundles was higher in the middle part of the second and third internodes in the ZUOErN plants (Supplementary Fig. S9).

The average height of ZUOErN3 and ZUOErN4 plants showed a significant increase, 10.6% and 10.7% (P=2.768 × 10⁻² and P=3.529 × 10⁻²), respectively, when compared with the
98.0 ± 3.19 cm height of ZH11 plants (Supplementary Table S1). Longitudinal sections showed that the longer stems of ZUOErN3 plants are due to elongated cells, compared with ZH11 plants (Fig. 2D).

Flag leaves are the main source organs that determine grain yield potential (Tan et al., 2012). The ZH11 plants had a flag leaf area of 33.40 ± 2.96 cm²; the flag leaf areas of ZUOErN3 and ZUOErN4 were 53.31 ± 10.42 cm² and 45.41 ± 6.36 cm² (P=3.089 × 10⁻⁴ and P=1.276 × 10⁻³), respectively (Fig. 3A, D). More importantly, the flag leaf angles did not change noticeably in ZUOErN3 and ZUOErN4 plants when compared with ZH11 (Fig. 1A). These results suggested that the ZUOErN plants may have a higher photosynthetic rate. In-depth characterization indicated that in the central part of the flag leaves, the number of large vascular bundles was significantly higher in ZUOErN3 and ZUOErN4 plants (11.89 ± 1.05 and 12.20 ± 0.84, respectively) when compared with the average of 10.25 ± 1.28 in ZH11 plants (P=1.115 × 10⁻² and P=1.208 × 10⁻³) (Fig. 3B, E). There were more small vascular bundles in ZUOErN3 and ZUOErN4 plants (46.75 ± 2.25 and 47.60 ± 0.89, respectively) than in ZH11 plants (42.25 ± 3.11) (P=5.076 × 10⁻³ and P=3.491 × 10⁻³) (Fig. 3B, E). The increased number of vascular bundles in the overexpression lines may facilitate the transport of photosynthetic products. Moreover, the ZUOErN plants showed much better developed vascular...
systems and mechanical tissues (Fig. 2B, E), which is crucial for high yield in rice.

The transgenic plants had longer root systems when compared with ZH11 plants in the flowering period (Fig. 3C). The total length of the root system in the ZUOErN3 plants was 21 853 ± 1719 cm, representing a 106.6% increase in comparison with the 10 578 ± 1643 cm of ZH11 per plant (P=1.200 × 10⁻³) (Fig. 3F). Denser and longer roots in transgenic rice plants may facilitate the absorption of water and nutrients, therefore contributing to a higher grain yield. In summary, these results suggested that the structural changes in the leaves, stems, and roots in the overexpression lines may facilitate the improved grain number and enhanced yield.

Expression of genes involved in grain number in ZUOErN plants

Plants overexpressing OsNAC2 showed increased grain number and higher yield; we therefore measured several yield-related genes, such as IPA1, DEP1, and Ghd7 in ZUOErN3 and ZUOErN4 plants. The qRT-PCR analysis revealed a significant up-regulation of IPA1 in ZUOErN3 and ZUOErN4 transgenic plants (Supplementary Fig. S7A). Previous studies have demonstrated that IPA1 increases grain number through the up-regulation of DEP1 (Lu et al., 2013). We therefore measured the expression levels of DEP1 as well and found that it was up-regulated by >4-fold in OsNAC2-overexpressing lines (Supplementary Fig. S7B). Furthermore, we found that the expression level of DTH8 was reduced by 24.5% and 29.0% in ZUOErN3 and ZUOErN4 plants, respectively, and the expression of Ghd7 was reduced by 36.3% and 39.8%, respectively (Supplementary Fig. S8). qRT-PCR analysis of OsNAC2-overexpressing lines showed no obvious alteration in the expression level of An-1, DST, HTD1, HTD2, and SP1 (Supplementary Fig. S8) that are related to grain number. These results suggested that OsNAC2 may control grain number and plant architecture through an IPA1-DEP1-related pathway.

OsNAC2-RNAi plants have small panicles

To examine the effect of decreased OsNAC2 expression, we constructed an RNAi vector against OsNAC2 driven by the native OsNAC2 promoter (Li et al., 2011). We transformed the construct into ZH11 plants and obtained 12 independent RNAi lines. Seven of these RNAi lines displayed a stunted phenotype (Fig. 4A; Supplementary Table S1) with panicles shorter than those of ZH11 plants (Fig. 4B, C). RT-PCR indicated a significant reduction of the OsNAC2 expression level in the RNAi plants (Fig. 4D). When compared with ZH11 plants, the panicle length of RNAi10 and 11 lines was 10.68% and 6.11% shorter (Fig. 4C), and the grain number was reduced by 19.71% and 10.79%, respectively (Fig. 4E, F). In addition, the number of both primary and secondary branches of the main panicles was reduced in the RNAi plants (Fig. 4G, H). However, no significant differences in tiller number, 1000 grain weight, or seed setting rate were observed in RNAi plants when compared with the WT (Supplementary Table S1). Taken together, the overexpression and RNAi experiments indicate that OsNAC2 controls multiple yield-related traits in rice plants, including panicle length, branch number, and grain number.

Overexpression of OsmiR164b leads to small panicles

Our results indicated that miR164b regulates OsNAC2 (Supplementary Figs S2, S3). To confirm further the regulation of OsNAC2 by miR164b and its effects on yield-related traits, an overexpression construct containing OsmiR164b driven by the maize Ubi1 promoter was generated and introduced into ZH11 plants (Zhonghua11-Ubi1-overexpression OsmiR164b, ZUOE164). Of the 18 transgenic lines obtained, 17 exhibited dwarfism and short panicles (Fig. 5A, D). The main panicle length was 18.38 ± 1.09 cm for ZUOE164-1 and 15.78 ± 2.73 cm for ZUOE164-2 plants, both of which significantly differed from the panicle length of ZH11 plants (Fig. 5B). The grain numbers also decreased from 160.3 ± 22.3 in ZH11 plants to 76.0 ± 14.5 and 60.3 ± 7.4 in ZUOE164-1 and -2 plants, respectively (Fig. 5C). The ZUOE164-1 and -2 plants had fewer primary and secondary branches (Supplementary Fig. S10). Using qRT-PCR, we found that miR164b expression levels increased in ZUOE164 plants (Fig. 5E) whereas OsNAC2 transcript levels decreased (Fig. 5F). Down-regulation of OsNAC2 leads to smaller panicles and fewer grains in plants overexpressing OsmiR164b or expressing OsNAC2-RNAi, which demonstrated that miR164b regulates panicle length and grain number by modulating OsNAC2 transcript levels.

The binding site for miR164 is highly conserved

Our study revealed that the overexpression of OsNAC2 with a mutated miRNA-binding site significantly enhanced the yield potential of rice plants. To determine whether a similar mutation had occurred under natural conditions or during domestication, we obtained the sequences of OsNAC2 from the genome sequences of 3000 rice accessions (Li et al., 2014). We identified 33 SNPs in the promoter and coding regions of OsNAC2 among 3000 rice varieties (Li et al., 2014; Alexandrov et al., 2015). However, we detected no mutation in the binding site of miR164b in OsNAC2 between positions 22 995 991 and 22 996 010 of chromosome 4 (Supplementary Table S3), suggesting that this sequence is evolutionarily conserved in rice. This observation explains why yield-enhancing alleles of OsNAC2 have not been found in rice cultivars.

Potential application of OsNAC2 in high-yield breeding

To evaluate the function and potential application of OsNAC2 in other rice varieties, we transferred the miR164b-resistant OsNAC2 transgene into Nipponbare (Nipponbare-UOErN, NUOErN) by backcrossing with recipient lines. The obtained NUOErN3 and NUOErN4 lines showed IPA traits such as increased tiller number, when cultivated in Guangzhou, Guangdong province, China (Fig. 6A). The grain weight per plant significantly increased from 10.36 ± 3.60 g in control plants to 21.49 ± 3.46 g and 18.27 ± 4.26 g in NUOErN3 and

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NUOErN4 plants, respectively (Fig. 6B). These results indicate that overexpression of OsNAC2 has a similar effect on increasing grain yield in the Nipponbare rice variety.

Yangyujing 3 is a widely used new rice variety with high yield and good grain quality. We produced Yangyujing 3 plants containing the miR164b-resistant OsNAC2 transgene by back-crossing with recipient lines (Yangyujing3-UOErN, YUOErN) and grew the plants in winter 2015 in Sanya, Hainan province, China. Statistical analysis of agronomic traits of these plants showed that YUOErN3 and YUOErN4 lines had improved architecture and enlarged panicles (Fig. 6C). The grain weight per plant also increased from 41.8 ± 10.01 g in control plants to 66.3 ± 12.44 g and 55.46 ± 9.10 g in YUOErN3 and YUOErN4 plants, respectively (P = 8.743 × 10 ^{-3} and P = 3.866 × 10 ^{-2}) (Fig. 6D). Taking these observations together, we conclude that expression of an miRNA-resistant OsNAC2 can be used to improve the yield in rice.

Discussion

Improving grain yield has been a primary goal for rice breeding. At the end of the last century, Japanese scientists and researchers at the International Rice Research Institute (IRRI) proposed the concept of super-high yield breeding and super rice varieties, and put these concepts into practice (Peng et al., 2008; Xu and Chen, 2016). In 1996, the Chinese Ministry of Agriculture initiated the Super Rice Project, aiming to improve rice yield by combining heterosis between subspecies and the establishment of ideal plant architecture (Qian et al., 2016; Xu and Chen, 2016). Recently, several super hybrid rice combinations have been cultivated by breeders, providing concrete evidence for the utility of this theory (Qian et al., 2016). Ideotype breeding or ideal plant architecture includes low tiller number, fewer or no unproductive tillers, high grain number per panicle, and thick and sturdy stems, as proposed by the IRRI (Peng et al., 2008; Miura et al., 2010; Lu et al., 2013).

IPA1/OsSPL14 affects these features and was identified a few years ago; an SNP in the coding region of IPA1 affects miR156 targeting and confers an ideal plant architecture (Jiao et al., 2010; Miura et al., 2010). IPA1 is regulated at the transcriptional and post-transcriptional levels (Lu et al., 2013; Wang et al., 2017; Zhang et al., 2017). IPA1 may be a ‘new green revolution gene’, as IPA-mediated traits can be fine-tuned by manipulation of IPA1 expression, which may further improve yield (Wang and Wang, 2017; Zhang et al., 2017).

In this study, the OsNAC2 overexpression plants had thicker stems, longer panicles, and increased grain number, which are characteristic of IPA, and showed significantly increased yield.

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Manipulating NAC2 expression improves rice architecture and grain yield

under field conditions. The expression level of both IPA1 and DEP1 was increased in ZUOErN plants. Our findings show that OsNAC2 is an important regulator of rice architecture and could be useful for breeding new high-yield varieties.

In this study, we provided evidence that OsNAC2 is a target gene of miR164b. Overexpression of miR164b-resistant OsNAC2 greatly enhanced grain number and rice yield. When OsNAC2 driven by the Ubi promoter was introduced in rice, there was no significant difference between the transgenic and the WT plants. However, when point mutations were introduced in the binding site sequence without any amino acid changes, plants transformed with the mutated OsNAC2 gene (ZUOErN) displayed improved grain number and enhanced grain yield in several planting seasons. An increased transcript level of OsNAC2 was observed in the ZUOErN lines. Furthermore, in OsmiR164b overexpression plants and OsNAC2-RNAi plants, the level of OsNAC2 transcript was decreased, and the panicle length and grain number were decreased as well. We therefore speculate that the expression of OsNAC2 is a critical factor for determination of grain number.

Traditional breeding programs largely rely on natural mutations and long-term artificial selection. Many agronomic traits are commercially important and have been characterized at the molecular level (Jin et al., 2008; Tan et al., 2008; Xue et al., 2008; Huang et al., 2009; Jiao et al., 2010; Li et al., 2010; Miura et al., 2010; Qian et al., 2016; Si et al., 2016). Naturally occurring yield-related alleles that affect miRNA-related functions have been identified for only a few genes (Jiao et al., 2010; Wang et al., 2012; Che et al., 2015; Duan et al., 2015; Gao et al., 2015). Using a whole-genome alignment of 3000 rice accessions (Li et al., 2014), we found that the binding site for miR164b in OsNAC2 showed a high degree of conservation, suggesting that natural variation in this site will not prove useful for improvement of rice yields using traditional breeding methods. Therefore, novel methods to create new rice varieties with higher, more stable yields are required. In this study, rice plants with significantly increased yield were created by introducing mutations into the miR164b-binding site of OsNAC2. Our research also provides a starting point for future investigation and applications of similar genes.

The OsNAC2 gene appears to play an important role in rice development. However, different functions have been ascribed to OsNAC2 in different studies. Ostill, a CaMV35S promoter activation-tagging mutant, in which the expression of OsNAC2 is significantly up-regulated, led to wider tiller angles and reduced plant height (Mao et al., 2007).

Fig. 5. Phenotypes of plants overexpressing OsmiR164b. (A) Morphologies in the mature stage. (B) Length of main panicles. (C) Grain number per main panicle. (D) Morphologies of main panicles. (E) OsmiR164b expression detected by qRT-PCR. (F) OsNAC2 expression detected by qRT-PCR. ZH-WT, wild-type Zhonghua 11. ZUOE164-1 and ZUOE164-2 are transgenic Zhonghua 11 plants overexpressing OsmiR164b driven by the maize Ubiquitin 1 promoter. Means ±SD are given in (C), (E), (G), and (H) (n=10). *P<0.05; **P<0.01 (t-test). Scale bars=10 cm.
However, when OsNAC2 (OMTN2) was overexpressed, driven by the Ubi1 promoter, the transgenic rice plants displayed no altered phenotypes, with the exception of hypersensitivity to drought at the reproductive stage (Fang et al., 2014). In addition, Chen et al. (2015) found that plants overexpressing OsNAC2 under the control of the CaMV35S promoter were shorter and that OsNAC2 regulates genes that are involved in the gibberellin biosynthetic pathway. In the present study, we overexpressed OsNAC2 with the native miR164-binding site under the control of the Ubi1 promoter, and found that neither OsNAC2 expression nor plant morphology was altered in transgenic plants. Nevertheless, plants expressing miR164-resistant OsNAC2 driven by the Ubi1 promoter showed improved plant architecture. Taken together, these results indicate that the function of OsNAC2 may be related to different expression patterns, or different insertion positions in the chromosome.

**Supplementary data**

Supplementary data are available at JXB online.

Table S1. The agronomic traits of transgenic and wild-type plants.

Table S2. Primers used for RT-PCR and plasmid construction.

Table S3. The SNP type of each rice accession from ‘The 3000 rice genomes project’ in the gene OsNAC2.

Fig. S1. Nuclear localization of the OsNAC2–GFP fusion protein in rice leaf sheath protoplasts.

Fig. S2. Morphologies of Zhonghua 11 and OsNAC2 overexpression plants.

Fig. S3. Location of the OsmiR164b-binding site in the OsNAC2 nucleotide sequence.

Fig. S4. Phenotype of ZUOErN transgenic lines in field conditions.

Fig. S5. Main processing quality traits in ZH-WT, ZUOErN3, and ZUOErN4 transgenic plants.

Fig. S6. Uniformity of panicles from single plants of ZH-WT and ZUOErN3 plants.

Fig. S7. Up-regulation of IPA1 and DEP1 in ZUOErN transgenic plants.

Fig. S8. Expression level of grain number-related genes in ZUOErN transgenic plants.

Fig. S9. Number of large and small vascular bundles in second and third internodes in the stem.

Fig. S10. Branch number per main panicle of OsmiR164b overexpression plants.

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