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ORIGINAL PAPER

A down-regulated epi-allele of the *genomes uncoupled 4* gene generates a *xantha* marker trait in rice

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Abstract

Key message **A γ-ray-induced *xantha* trait is epigenetically controlled by the *genomes uncoupled 4* gene with enhanced promoter segment methylation and down-regulated expression in rice.**

Abstract For easy testing and to increase varietal purity, a *xantha* mutation (*xnt*), which turns plants yellow and makes them visually distinguishable from normal green rice, has been generated and bred into male sterile lines for hybrid rice production. The *xnt* locus was previously fine mapped to a ~100-kb interval on chromosome 11, but its identity was unknown. In this study, *xnt* was further narrowed down to a 57-kb fragment carrying eight opening reading frames (ORFs). All eight ORFs had identical genomic sequences and all but ORF2 (*genomes uncoupled 4*, *OsGUN4*) had similar transcript abundance in the *xantha* mutant Huangyu B (HYB) and its parental variety Longtefu B (LTB). The expression of *OsGUN4*, however, was significantly reduced in HYB compared with LTB in terms of both transcript abundance (0.2 % that of LTB) and expressed protein level (barely detectable in HYB but greater than the heat shock protein reference in LTB). Therefore, *OsGUN4* was identified as the candidate gene underlying the *xantha* trait. The function of *OsGUN4* in the *xantha* phenotype was confirmed by identification and characterization of new allelic *OsGUN4* mutations. Comparative bisulfite genomic sequencing of *OsGUN4* revealed increased methylation in a promoter region in the mutant, and the correlation between increased methylation and the *xantha* phenotype was further verified by demethylation treatment. In summary, we have identified an epi-allele of *OsGUN4* as the causal gene of the *xantha* marker trait and revealed that enhanced methylation in its promoter down-regulated its expression in rice.

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Introduction

Hybrid rice has played an important role in rice production in China during the past 30 years and has recently become popular in a number of rice-producing countries such as India and the United States. High varietal purity is essential for hybrid rice to manifest its heterosis in production. Fluctuations of environmental and climatic conditions during hybrid seed production often cause partial fertility reversion of male sterile (MS) lines, resulting in various degrees of MS seed contamination in commercial hybrid seed batches (Shu et al. 1996). The two-line hybrid rice system, which is based on photoperiod- or temperature-sensitive genic male sterile (P/TGMS) lines (Huang et al. 2014), is particularly susceptible to such fluctuations because unusual low temperatures during hybrid seed production can revert the fertility of P/TGMS lines. Consequently, it has been a great challenge to maintain high varietal purity

(Si et al. 2011). To increase, assure, and rapidly test varietal purity, two types of marker traits have been incorporated into commercial MS lines used in hybrid rice production. The first includes various recessive non-green leaf color traits, e.g., *chlorina* (Dong et al. 1995), *xantha* (Zhou et al. 2006a), green-revertible albino seedlings (Wu et al. 2003, 2011), and purple plants (Mou et al. 1995). The second consists of conditional recessive lethality, e.g., the bentazon susceptibility mutation (Zhang et al. 2002; Wang et al. 2012). Commercial hybrid rice varieties based on MS lines with such marker traits have already been used in commercial production (Cao et al. 1999; Yu et al. 2000; Bao et al. 2006; Shen et al. 2004, 2007; http://www.ricedata.cn/variety/varis/605067.htm; http://www.ricedata.cn/variety/varis/601809.htm).

The *xantha* marker trait of Huangyu B (HYB) was generated by ^{60}Co γ-irradiation of dried seeds of Longtefu B (LTB), an *indica* rice and the maintainer line of the widely used commercial cytoplasmic male sterile (CMS) line Longtefu A (Zhou et al. 2006a). HYB has significantly reduced levels of chlorophyll (Chl) *a* and *b*, carotenoids (Car), and higher ratios of Chl *a/b* and Car/Chl compared with LTB (Zhou et al. 2006b). HYB retains a high photosynthetic rate (Zhou et al. 2006b) and is efficient in solar energy utilization (Wu et al. 2007a). The *xantha* trait was subsequently bred into CMS lines such as Huangyu A (Zhou et al. 2006a; Shen et al. 2007) and Jiazhe 91A (Fu et al. personal communication). A few Chl mutants, such as *Chlorina-1* and *Chlorina-9* (Zhang et al. 2006), *Yellow-Green Leaf 1* (Wu et al. 2007b), and *Yellow-Green Leaf 2* (Chen et al. 2013), have similar phenotypes to that of HYB, often with yellow-green leaves due to reduced Chl *a* and *b* contents. However, the *xantha* mutation (*xnt*) in HYB is postulated to differ from known leaf color mutations as it was mapped to a ~100-kb interval on the short arm of chromosome 11, where no cloned chlorophyll-deficient mutant genes are located, although its identity has yet to be revealed (Chi et al. 2010).

It has been well documented that epigenetic regulation of gene expression plays important roles in evolution, transposon silencing, and genomic imprinting (Miura et al. 2009; Martienssen and Colot 2001; Law and Jacobsen 2010). It is also known that heritable mutations can be epigenetically controlled, though few examples have been characterized in plants, especially in crops; for instance, only four epigenetic mutants have so far been characterized in rice (reviewed by Chen and Zhou 2013).

In this study, we discovered that an epi-allele of the rice *genomes uncoupled 4* (*OsGUN4*) gene underpins the *xantha* phenotype. Thus, we not only experimentally proved that *OsGUN4* is involved in chlorophyll and carotenoid metabolism, but also found an intriguing example of epigenetic control of an important agronomic trait.

Materials and methods

Development of mapping and mutagenized populations

For fine mapping of the *xantha* mutation, Huangyu A was crossed to two wild-type (WT) restorer lines, Zhongke 600 and #10–1760, with normal green leaves in 2009. Two F_2 populations were subsequently produced in 2011 through self-pollination of F_1 plants in 2010. *xantha* F_2 seedlings were identified in seedling beds at the five-leaf stage and transplanted into paddy fields at the Experimental Farm of Zhejiang University on Zijingang Campus, Hangzhou, China. Adult plants showing the *xantha* phenotype were used for gene mapping and 12 plants were further developed into higher generations for characterization.

To identify allelic *xnt* mutations, dried seeds of the TGMS line GS113 (Zhang et al. 2014) were mutagenized by 300-Gy ^{60}Co γ-irradiation at a dose rate of 5 Gy min^{-1} at the Irradiation Centre of Zhejiang Academy of Agricultural Sciences. About 5,000 M_1 plants were grown in the Experimental Farm of Jiaxing Academy of Agricultural Sciences from November 2012 to March 2013 in Lingshui, Hainan Province, China. GS113 headed on late February 2013 and was partially fertile because of the low temperature and short-day climate conditions in February.

From ~25 kg M_2 seeds of GS113 bulk-harvested from M_1 plants, 3 kg was randomly sampled and grown in parallel with Huangyu B to produce hybrid seeds in Anji, Zhejiang Province. The paddy field experiment was set up according to conventional practices in hybrid rice seed production, except that the ratio of the MS line (M_2 of GS113) to the parental line (Huangyu B) was reduced to 6:2 to achieve sufficient out-crossing of MS plants (Fig. S1a). About 25 days after flowering, panicles of GS113 M_2 plants filled with M_2F_1 seeds (with Huangyu B) were harvested and dried under sunlight to seed moisture of ~14 %. On-panicle seeds were imbibed in water for 2 days, germinated under temperatures around 30 °C, and subsequently sown in panicle plots in pre-prepared seedling beds. From 10 days after sowing, the seedling beds were visually examined for seedlings with the *xantha* phenotype (Fig. S1b).

Gene mapping and genomic sequencing

To narrow down *xnt* within the ~100-kb region on chromosome 11 delimited by Chi et al. (2010), a total of 24 potential single nucleotide polymorphic (SNP) and 15 insertion/deletion (indel) markers were identified by comparing the genome sequences of *japonica* Nipponbare (http://ensembl.gramene.org/Oryza_sativa/Info/Index/) and *indica* 9311 (http://rise.genomics.org.cn/rice/link/download.jsp). Primers were designed to amplify fragments

encompassing the SNPs and indels for genotyping the F_2 *xantha* plants (Table S1).

To genotype the F_2 plants, DNAs were extracted from leaf tissues of *xantha* plants using the CTAB method. Indel markers were revealed using a standard PCR program with an annealing temperature of 55 °C followed by electrophoresis in 8 % polyacrylamide gels and silver-staining according to Liu et al. (2007). Gels were documented using the Versa Doc Imaging System Model 3000 (Bio-Rad, Inc., USA). SNP detection was performed using high-resolution melting (HRM) curve analysis according to Tan et al. (2013). Briefly, PCRs were performed in a 10-μL volume with 25 ng DNA, 5 μL 2× Master Mix (containing PCR buffer, $MgCl_2$, dNTPs, Taq DNA polymerase), 0.5 μL each of forward and reverse primer (10 μM/μL), and 1 μL LcGreen Plus (Idaho Technology Inc, USA). One drop of mineral oil was added to each PCR to prevent solution evaporation. The following program was used for amplification: 94 °C for 5 min; 36 cycles of 94 °C for 30 s, 55 °C for 30 s, 72 °C for 1 min; followed by 72 °C for 5 min. After amplification, the PCR plates were scanned with an HRM LightScanner (Idaho Technology Inc., USA) and analyzed with the proprietary software Call IT TM 2.0 according to the LightScanner Operator's Manual (Idaho Technology Inc., USA).

After *xnt* was restricted to a ~57-kb region, overlapping genomic fragments of the whole region were amplified using 38 pairs of primers (positions and sequences are shown in Fig. S4) using genomic DNAs extracted from 4-week-old rice seedlings. All PCRs used the following cycling conditions: 10 min at 95 °C; 36 cycles of 30 s at 94 °C, 30–120 s at 55 °C (the exact extension time was set according to length of an amplicon) and 60 s at 72 °C; 6 min at 72 °C. The PCR products were detected in a 1 % agarose gel in 0.5× TBE buffer, excised from the gel for purification using the DNA Gel Extraction Kit (Axy-Gen, China), and then sequenced at Nanjing Genscript Biotech. Co. Ltd. (Nanjing, China).

Quantitative RT-PCR and Western blotting

For quantitative RT-PCR (qRT-PCR) analysis, total RNAs were extracted from rice leaf tissue at the four-leaf stage using the Qiagen Spin Plant RNA Mini Kit (Qiagen, Germany). cDNAs were reverse transcribed from 1 μg total RNA using the oligo-dT_{18} primer and GoScript™ Reverse Transcription System Kit (Promega, USA) according to the manufacturer's instructions. qRT-PCRs were performed using a SYBR Green GoTaq® qPCR Master Mix kit (Promega, USA). One primer pair each was designed for qRT-PCR analysis of the eight ORFs within the *xnt* locus (qORF1–8, Table 1). All qRT-PCRs were performed in 10 μL volumes with ~25 ng cDNA in 1× GoTaq® qPCR Master Mix (containing PCR buffer, $MgCl_2$, dNTPs, Taq DNA polymerase), and 0.4 μM of each primer. The following program was used for all qRT-PCRs: 10 min at 95 °C, 40 cycles of 30 s at 94 °C, 30 s at 55 °C, and 60 s at 72 °C. Relative gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method with the rice *Actin* gene as the internal standard (Livak and Schmittgen 2001).

For Western blot analysis, proteins were extracted from 100 mg of plant tissue of 4-week-old rice seedlings. The tissues were homogenized in liquid nitrogen and resuspended at 65 °C for 60 min in 800 μL buffer (containing

Table 1 Primers used for quantitative real-time PCR analysis and bisulfite sequencing of *OsGUN4*

Code	Forward primer (5′→3′)	Reverse primer (5′→3′)	Amplicon (bp)
1. Primers used for qRT-PCR analysis			
qORF1	ACGGCTATCAGCCAGCATCAGT	GCTGCCAAGGTGTTGAGGCCA	263
qORF2	TTACCGGCAGGCCGACGAGA	TGCCCAGGAGCTGTGTCCCT	377
qORF3	ACCAGATCAACCTGCTCAAG	CATTTCACGCACAAGGACAC	143
qORF4	GATGTGATCATGGCTGAGAGG	AACTACGACACCATTGAGCTC	147
qORF5	GACTTGACCAAA- ACTGAAGCG	GATGCCTTGCCATATCGAAAATC	137
qORF6	GAATT- CTCAAAGGTCTCCGGG	AGTCAATTGCTTTATCCGGGAG	135
qORF7	GGTAAAAGAGATGGAGACAGGG	TCTGGGAATTCAGTAGCAAG	149
qORF8	CGCTGTTAGTCCTGTTAGTCG	AGGTGGTTCACTTTGTTTGC	358
Actin	CTTCATAGGAATGGAAGCTGCGGGTA	CGACCACCTTGATCTTCATGCTGCTA	196
2. Primers used for bisulfite sequencing of *OsGUN4*			
me1	G TTTAGGGGTATAGAAATGATTG	ACAACACACAAATAAAAAAACTT	245
me2	TTAAGTTTTTTTATTTGTGTGTTGT	TTAAAACACACTCCCAACC	207
me3	GGTTGGGAGTGTGTTTTAA	CAAAATAATACRCATAACAACAC	374
me4	TTGTTATGYGTATTATTTTGTG	AAACCAAACATTACTCATCCTA	240
me5	TTAGGATGAGTAATGTTTGGTT	CATAACCTAATAAAATTTATTCCCT	291

2 % SDS, 50 mM Na_2CO_3, 12 % sucrose, 50 mM DTT and 2 mM EDTA, pH 8.0) with 0.1 mM phenylmethylsulfonyl fluoride (PMSF) and protein inhibitors (Sigma). The suspensions were then centrifuged to remove cell debris. The protein concentration was determined with BCA reagent (Thermo Scientific, USA) according to the manufacturer's instructions. The OsGUN4-specific antibody was generated by selecting a specific epitope, FTRFFIRVGWMKKL, using the PepDesign software (Cao et al. unpublished) and then verifying its uniqueness in the rice whole proteome by a BlastP search (Altschul et al. 1997) against the rice database (http://rice.plantbiology.msu.edu/). Anti-OsGUN4 polyclonal antibodies were generated by immunizing healthy rabbits using KLH-conjugated synthetic peptides as antigens. The peptide conjugations, immunizations, and antiserum purifications were carried out by BPI (Beijing Protein Innovation Co., Ltd., Beijing, China). After segregation by SDS-PAGE on an 8 % gel, the proteins were immunoblotted according to Zhao et al. (2013), using the heat shock protein Os09g0482600 as an internal reference (Li et al. 2011).

Bisulfite sequencing and demethylation treatment

For DNA methylation analysis, ~1 μg genomic DNA was treated with sodium bisulfite using the EZ DNA Methylation Kit (Zymo Research, Los Angles, USA) following the manufacturer's instructions. Five primer pairs (me1–me5, Fig. 2a; Table 1) were designed for amplification of *OsGUN4* from bisulfite-treated genomic DNAs using the Methyl Primer Express software v1.0 (Applied BioSystems, USA). PCRs were performed in 50 μL volume with 50 ng treated DNA and 1× EpiTaq Buffer (without $MgCl_2$), 2.5 mM $MgCl_2$, 0.3 mM dNTP mixture, 0.4 μM of each primer, and 1.25 U EpiTaq HS polymerase (Takara, Japan). The PCR products were purified and cloned into the pGEM-T easy vector (Promega, USA). At least 25 clones were sequenced for each amplicon, and the sequencing results were analyzed with the Kismeth software (Gruntman et al. 2008).

DNA demethylation treatment was performed as described by Miura et al. (2009) with slight modifications. Briefly, dehulled seeds were sterilized with 70 % (v/v) ethanol for 5 min and 1 % NaClO for 10 min, and washed at least three times in sterile distilled water. The seeds were placed on MS medium (Murashige and Skoog 1962) with or without 70 mg/L 5-aza-2′-deoxycytidine (5-aza-dC), grown under dim light at 28 °C for 6 days, and then transferred to a growth chamber (16 h of 850 μmol m^{-2} s^{-1} light/8 h of dark, at a constant 25 °C) until leaf color phenotypes were obvious.

Statistical and bioinformatics analysis

Quantitative RT-PCRs were performed with 3–6 independent biological replications. To test the significant differences, *F* tests were used and followed by Student's *t* test with a *P* value <0.05 as the threshold for significant difference. PCR primers were designed on the NCBI website using the Primer 3.0 program (http://www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi?LINK_LOC=BlastHome). Except where indicated, the Nipponbare genome sequence was used for reference information (New Gramene Release 41). Sequences were analyzed using the Bioedit (http://www.mbio.ncsu.edu/BioEdit/bioedit.html) and Editplus (http://www.editplus.com/).

Results

Identification of *OsGUN4* as the candidate gene underlying the *xantha* trait

A total of 24 SNP and 15 indel markers were identified between 93-11 and Nipponbare within the 100-kb region containing the *xnt* (Chi et al. 2010), and were subjected to polymorphism test between Huangyu A and Zhongke 600, and between Huangyu A and #10-1,760. Three indel (IDS1, 3, 6) and four SNP (SNP5, 10, 18, 24) markers were identified as polymorphic between the former two lines, and four indel (IDS1, 3, 6, 7) and four SNP (SNP5, 10, 20, 24) markers between the latter two (Table S1). These polymorphic markers were subsequently used for genotyping of *xantha* F_2 plants of the two crosses. Initially, a total of 1,899 *xantha* F_2 plants (1,088 of Huangyu A/Zhongke 600 and 811 of Huangyu A/#10-1,760) were genotyped using IDS1 and SNP24 (Fig. 1a, b). In the Huangyu A × Zhongke 600 population, only two plants were identified as heterozygous for SNP24 (Fig. 1a), while 18 and one heterozygous plants were detected for IDS1 and SNP24, respectively, in the Huangyu A × 10-1,760 population (Fig. 1b). These recombinant plants were further analyzed using inward markers from both sides, one by one, until there were no more recombinants (Fig. 1a, b). Ultimately, *xnt* was delimited to a 57-kb interval between IDS7 and SNP18 (Fig. 1c).

The 57-kb fragment contained eight open reading frames (ORF1–8, Fig. 1c). ORF2 and ORF6 were annotated as *genomes uncoupled 4* (*OsGUN4*, Os11g1267000) and the ATP-dependent chloroplast protease ATP-binding subunit clp A (*OsCLP*), respectively; hence, their mutations seemed most likely to be responsible for the *xantha* phenotype. Sequencing analysis, however, showed no difference in either gene between HYB and LTB, suggesting that mutation(s) in other ORFs or intergenic regions might have caused the *xantha* phenotype. Therefore, the whole 57-kb region was subsequently sequenced, but again no single nucleotide difference was found between HYB and LTB, although there were numerous differences between HYB and Nipponbare (Fig. S3).

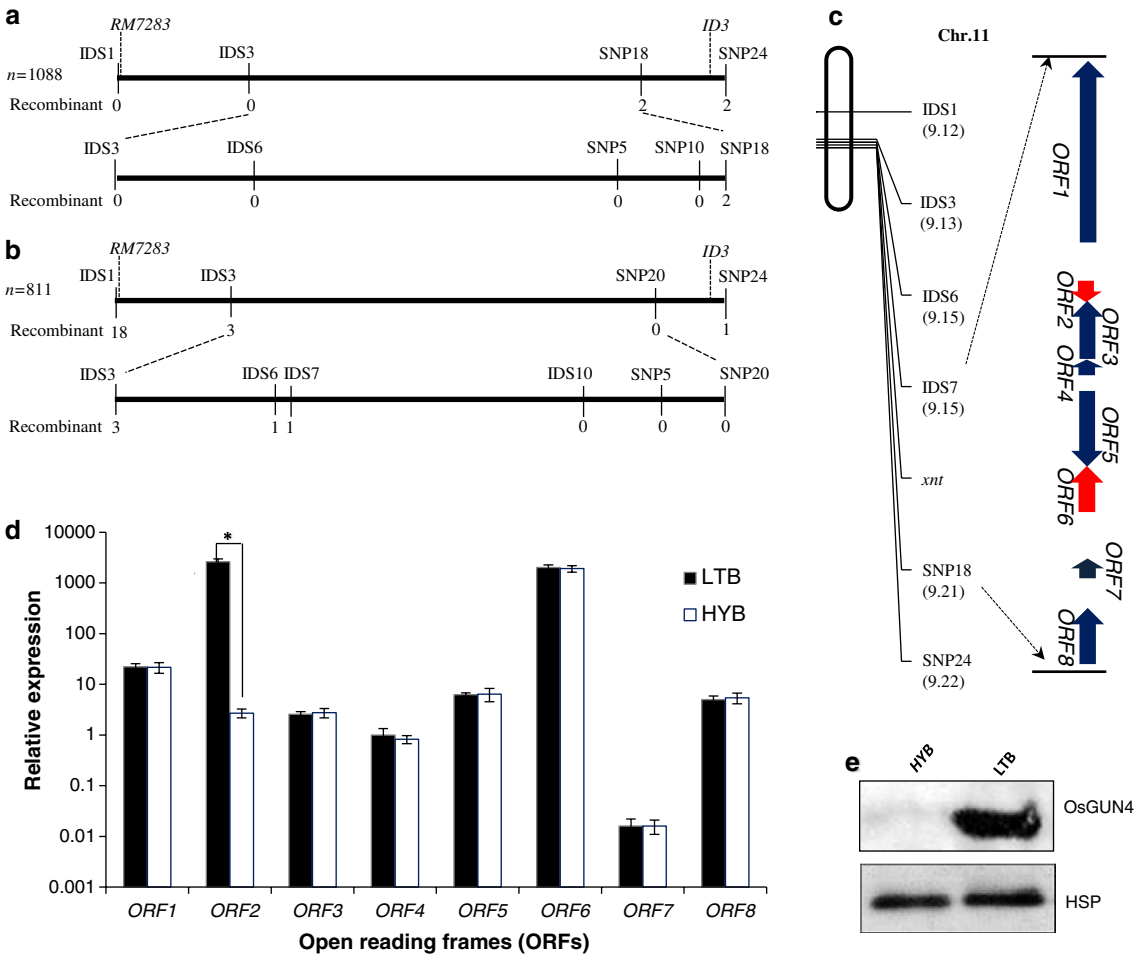

Fig. 1 Fine mapping (**a**, **b**) and candidate gene analysis (**c**–**e**) of the *xantha* mutation (*xnt*). **a**, **b** Genotyping of *xantha* F_2 plants of Huangyu A × Zhongke 600 and Huangyu A × #10-1760 using SNP and indel (IDS) markers. *n* total number of plants; the number of heterozygous plants is given below each marker. **c** Schematic presentation of the combined mapping results (*left*; in *parentheses* are the genomic positions of each marker in mega base pairs) with the *open reading frames* (ORFs) annotated within the delimited interval (*right*); the two ORFs potentially involved in chlorophyll metabolism are highlighted in *red*. **d** qRT-PCR analysis of the eight *ORFs* in the 57-kb region; data from Huangyu B (HYB) and Longtefu B (LTB) marked with an *asterisk* are significantly different ($P < 0.05$); the expression level of *ORF4* in LTB was set as 1. **e** Western blot assay of OsGUN4 expression with heat shock protein (HSP) as an internal reference

These unexpected results prompted us to examine whether there was any difference in gene expression. Thus, we analyzed the relative transcript abundance of the eight ORFs using qRT-PCR. *OsGUN4* and *OsCLP* showed hundreds of times greater abundance than the other *ORFs* in LTB, but *OsGUN4* was significantly down-regulated in HYB, having only ~0.2 % of the transcript of its parental line LTB (Fig. 1d). The adjacent ORFs up- or down-stream of *ORF2*, in contrast, showed no significant difference in transcript abundance between HYB and LTB (Fig. 1d).

The protein level of OsGUN4 was also analyzed by Western blotting. The results indicated that HYB had a barely detectable level of OsGUN4 protein in its leaf tissue while LTB had an OsGUN4 level even greater than that of the internal reference heat shock protein (Fig. 1e).

The results of gene mapping and expression analysis, and the phenotypic resemblance of HYB to the *GUN4* mutant *gun4-1* in Arabidopsis (Larkin et al. 2003), suggested that the *xantha* phenotype was likely to be underpinned by an as yet unknown *OsGUN4* mutant allele.

Identification and characterization of allelic *OsGUN4* mutations

To identify allelic mutations of *OsGUN4*, we generated an M_2 population of the TGMS line GS113 and, when it was male sterile, crossed it to HYB to produce M_3F_1 seeds (Fig. S1a). Theoretically, when a green GS113 M_2 plant carrying a recessive *OsGUN4* allele in a heterozygous state is crossed to HYB, normal green and *xantha* seedlings are

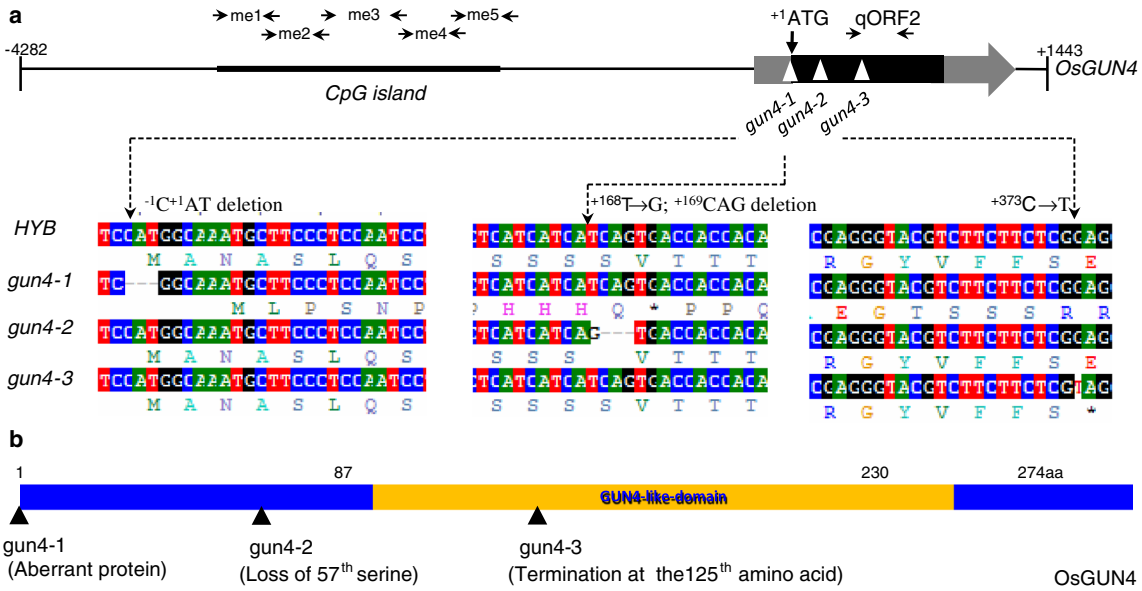

Fig. 2 Analysis of the rice *GUN4* gene and its mutations. **a** Schematic diagram of the *OsGUN4* locus. Primers used for bisulfite sequencing (me1–5) and qRT-PCR (qORF2) are shown above the gene; nucleotide positions are relative to the translation start site (+1). The predicted CpG island is shown with a bold line. 5′ and 3′ untranslated regions are shown in *gray boxes* and the coding region in a *black box*. Mutation sites for the three *gun4* mutant alleles are indicated with empty triangles; details of nucleotide changes and their predicted effects on translation are shown below the locus. **b** The structure of OsGUN4 with a GUN4-like domain in the center; the mutation sites and their predicted effects on the protein are shown

expected in its M_3F_1 progenies. Therefore, we screened for such M_3F_1 *xantha* seedlings in ~30,000 M_3F_1 panicle plots (with 30–50 seedlings each, Fig. S1b). Four plots were identified that were segregating for *xantha* and WT seedlings (Fig. S1c, and data not shown). Because of the low and decreasing temperatures in late October, all seedlings of these four panicles were transferred into a growth chamber and grown for 2 weeks before being transplanted into paddy fields in Lingshui, Hainan.

Microsatellite markers that were polymorphic between GS113 and HYB were analyzed in the *xantha* seedlings and the results confirmed that they were hybrids of GS113 and HYB (data not shown). To reveal whether there were *OsGUN4* mutations in these *xantha* seedlings, we sequenced the *OsGUN4* locus for one seedling from each of the four panicles. Three *OsGUN4* mutant alleles were identified: one with a 3-bp (CAT) deletion starting from the −1 bp locus (hereafter *gun4-1*, observed in the *xantha* seedlings in two panicle rows), one with a 3-bp (CAG) deletion at the +169–171 bp locus (*gun4-2*), and one with a C → T transition at the +373 bp locus (*gun4-3*) (Fig. 2a). The two panicles that produced *gun4-1* seedlings likely came from the same M_2 GS113 plant.

Bioinformatics analysis showed that the *gun4* mutations had different effects on the function of OsGUN4. The *gun4-1* allele caused loss of the ATG start codon and a frameshift forming a new open reading frame from $^{+8}$ATG to $^{+127}$TAA, resulting in a new and greatly shortened aberrant protein completely different from OsGUN4; hence, it could be considered a loss-of-function allele. The *gun4-2* mutation resulted in the removal of the 57th serine but did not cause frameshift (Fig. 2b). The C → T mutation produced a premature stop codon at the 125th amino acid and hence the *gun4-3* allele is expected to encode a truncated GUN4 protein (Fig. 2b).

Because there was a barely detectable level of OsGUN4 protein in the *xantha* mutant HYB (Fig. 1e), the phenotypes of its F_1 hybrid plants were expected to be dependent on the other allele. Indeed, this was the case in the M_3F_1 plants; *gun4-1*/*xnt* plants retained the *xantha* phenotype throughout their growth period and showed substantially weaker growth than WT hybrid plants (Fig. S1d). The *gun4-2*/*xnt* plants turned into normal leaf color approximately from the 5th leaf (data not shown). The *gun4-3*/*xnt* plants also turned green when examined about 1 month after transplanting in the paddy field (the exact timing of color transition was not determined since the experiment was carried out in the breeding nursery in Hainan, which is far from Hangzhou). Seeds of the survived *gun4-1*/*xnt* F_1 plants were harvested and grown into F_2 seedlings; while all seedlings showed yellow leaves similar to HYB for the first three leaves, some (~1/4) stopped growing and eventually died at seedling stage (Fig. S2a). Analysis by HRM indicated the presence of three genotypes, i.e., homozygous *gun4-1* (to

which all died seedlings belonged), homozygous *xnt* (*epi-GUN4*), and heterozygous seedlings (Fig. S2b).

These results unambiguously confirmed the hypothesis that the *xantha* phenotype of HYB is underpinned by a subtle allele of *OsGUN4*.

Increased methylation inhibited *OsGUN4* transcription

The fact that HYB had an *OsGUN4* DNA sequence identical to that of LTB plants but a significantly reduced expression level in terms of both transcript abundance and translated protein (Fig. 1d, e) suggested that the *xantha* trait of HYB might be regulated in an epigenetic manner. Bioinformatics analysis indicated that there was only one CpG island in the promoter region (−2,901 to −1,624 bp) across the whole *OsGUN4* locus (Fig. 2a). Hence, this fragment, amplified using primers me1–5 (Table 1; Fig. 2a), was subjected to bisulfite sequencing to detect possible epigenetic marks. Comparative analysis indicated that the methylation level and pattern of HYB differed from that of LTB in a 374-bp region within the CpG island (−2,497 to −2,124 bp, Fig. 3a). Compared with LTB, the methylation level of HYB at CHG sites was increased to 60.7 % (vs. 42.3 % in LTB), while the methylation levels at CG (97.9 vs. 96.4 %) and CHH (30.9 vs. 28.6 %) sites were similar between HYB and LTB (Fig. 3b). Remarkably, the $^{-2386}$C was completely methylated in HYB but was not methylated at all in LTB (Fig. 3a). Further analysis indicated that an antioxidant response element (ARE) existed in the core part of this 374-bp region (Fig. 3a, boxed), suggesting that this particular fragment may play an important role in the expression regulation of *OsGUN4*.

To examine whether the epigenetic status was transmitted to offspring plants of HYB, we also analyzed five F_3 *xantha* lines derived from the cross Huangyu A × Zhongke 600. Similar methylation patterns and levels to HYB were observed in these lines, i.e., complete methylation of the $^{-2386}$C (Fig. S3a) and increased methylation levels

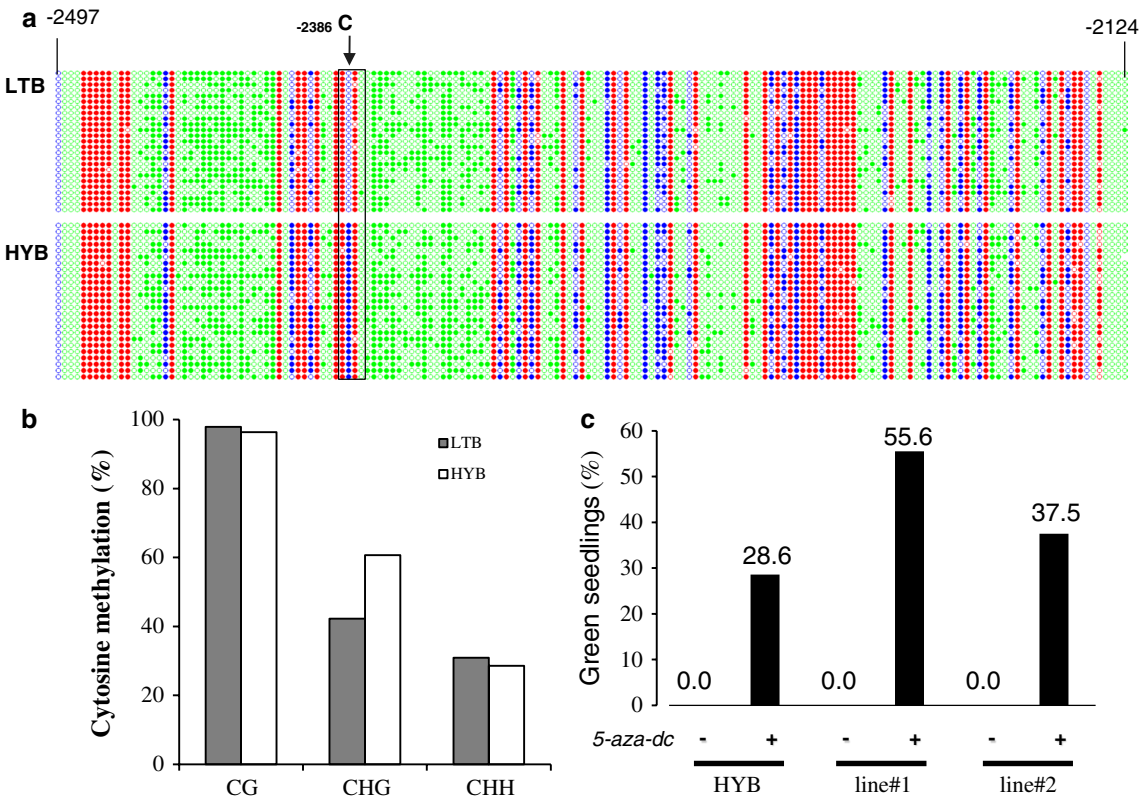

Fig. 3 DNA methylation and demethylation analysis of the *OsGUN4* locus. **a** A *dot plot* comparison of the methylation profiles of Longtefu B (LTB) and its *xantha* mutant Huangyu B (HYB) in the promoter region 2,497–2,124 bp upstream of the translation start site revealed by bisulfite sequencing. Cytosines in the form of CG are marked in *red*, CHG in *blue*, and CHH in *green*, where H = A, C, or T; *filled* and *empty circles* denote methylated and unmethylated cytosines, respectively. The *boxed* regions represent an antioxidant response element (ARE, predicted by Web Promoter Scan Service_ATF) in the promoter; the cytosine at position −2,386 was completely methylated in HYB but not in LTB (shown with an *arrow*). **b** Comparison of cytosine methylation rates between LTB and HYB for CG, CHG, and CHH sites. **c** Rates of green seedlings grown from seeds of *xantha* genotypes (HYB and its two derivative lines) treated with the inhibitor of DNA methylation 5-aza-dC (+)

(54–65 %) at the CHG sites (Fig. S2b). These results not only supported a link between increased methylation and the *xantha* phenotype, but also demonstrated that the methylation change was meiotically heritable.

To further study the correlation between methylation and the *xantha* phenotype, we treated seeds of HYB and two other *xantha* lines derived from Huangyu A × Zhongke 600 with 5-aza-2′-deoxycytidine (5-aza-dC), an inhibitor of DNA methylation (Chang and Pikaard 2005). While all non-treated seeds produced seedlings with the *xantha* phenotype, green leaf seedlings, ranging from 28 % in HYB to 55 % in one of its progenies (Line #1), were observed in all populations from treated seeds (Fig. 3c). These observations further supported the notion that the *xantha* phenotype of HYB was caused by the down-regulation of *OsGUN4* via increased methylation.

Discussion

In the present study, we aimed to determine the gene underpinning the *xantha* marker trait in the rice variety Huangyu B and identified a down-regulated epi-allele of *OsGUN4* to be responsible for the phenotype. This not only experimentally demonstrates that *OsGUN4* plays a role in chlorophyll and carotenoid metabolism in rice, but also provides a fascinating example of an epigenetically regulated agronomic trait, which might have been induced by gamma rays.

GUN4 and its function in chlorophyll biosynthesis

The GUN4 protein has been well studied in Arabidopsis and to a lesser degree in *Chlamydomonas reinhardtii* (Formighieri et al. 2012). Although not absolutely essential for chlorophyll biosynthesis (Larkin et al. 2003; Peter and Grimm 2009; Adhikari et al. 2011), GUN4 stimulates the activity of Mg-chelatase, which catalyzes the first committed step in chlorophyll biosynthesis by binding the CHLH subunit of the enzyme, the substrate, and the product of the reaction, and its expression is tightly correlated with the chlorophyll biosynthetic activity under diurnal growth conditions (Peter and Grimm 2009). In *Arabidopsis thaliana*, a number of *gun4* mutants have been identified and characterized. Depending on the mutation effect, *gun4* mutants have phenotypes ranging from albino to pale green to chlorotic (yellow-green) leaves under photoperiodic growth conditions (Larkin et al. 2003; Peter and Grimm 2009; Adhikari et al. 2011). In rice, little was known about GUN4 and its function. The present study is the first report on the identification of OsGUN4 and its phenotypic effect. HYB has Chl *a* and *b* reductions of 57 and 77 %, respectively (Zhou et al. 2006b), and its yellow plant phenotype is similar to that of Arabidopsis *gun4-1* (Larkin et al. 2003), which is consistent with the role of OsGUN4 in chlorophyll accumulation in rice under normal growth conditions.

Loss-of-function mutations can severely impair chlorophyll biosynthesis and plant growth; for instance, the *Arabidopsis* T-DNA insertion null mutant line *gun4-2* is very small with white or yellow leaves (Larkin et al. 2003). In the present study, we identified three new mutant *gun4* alleles: *gun4-1*, *gun4-2*, and *gun4-3* (Fig. 2a). Based on bioinformatic analysis, *gun4-1* is a loss-of-function mutation. The weaker growth (than HYB) of *gun4-1/xnt* F_1 hybrids (Fig. S1d) and the death of homozygous *gun4-1* F_2 seedlings (Fig. S2a) suggested that OsGUN4 is indispensible for rice growth under natural conditions; further studies will disclose its role and mechanism in chlorophyll biosynthesis in rice. Conversely, the *gun4-2* and *gun4-3* mutations had a less severe impact on chlorophyll synthesis and plant growth, such that *gun4-2/epi-GUN4* and *gun4-3/epi-GUN4* hybrids exhibited the *xantha* phenotype only at the seedling stage. The change of these hybrids from *xantha* to normal green indicated that gun4-2 and gun4-3 mutant proteins had normal functions for chlorophyll biosynthesis in adult plants, though further work is needed to confirm these observations.

Although this study did not address the molecular mechanism by which OsGUN4 works, the revelation that it is the causal gene of the *xantha* marker trait and the generation of new mutant allele *gun4-1* sets a sound basis for further characterization and functional analysis of OsGUN4. Because *gun4-2* and *gun4-3* plants were expected to turn green faster than their hybrids with HYB, we inferred that they would not be suitable marker traits and did not harvest seeds from the F_1 plants. As the experiment went on, it became increasingly clear that they would be excellent genetic resources for functional analysis of different parts of OsGUN4.

Characteristics of *epi-GUN4* allele

The role of epigenetic processes in development is now well documented. However, studies on the transgenerational inheritance of epigenetic modifications are still in their infancy, and the extent to which epigenetic variation contributes to phenotypic variation in plants remains unknown because only a dozen epigenetically controlled, meiotically inheritable mutant alleles (epi-alleles) have been identified in plants (reviewed by Weigel and Colot 2013). In the present study, we identified an *epi-GUN4* allele underpinning the *xantha* marker trait and demonstrated that the epi-allele also has its own characteristics, thus providing another intriguing example of epigenetic control of plant variation.

Most epi-alleles identified initially were associated with an adjacent repetitive element or a transposon, e.g., those

identified in maize (reviewed by Richards 2006) and the first epigenetic mutant in rice, *Epi-d1* (Miura et al. 2009). However, more recently identified epi-alleles seem not to be related with cis-acting elements, e.g., the epigenetically regulated allele *OsSPL14*^{WFP} (Miura et al. 2010), the epimutation *abnormal floral organ* (*afo*) (Wang et al. 2010), and the *Epi-df* allele of *FIE1* (Zhang et al. 2012), all found in rice. The overall methylation level in the CpG island of the *epi-GUN4* allele was not different from the WT allele, but the methylation level was substantially increased in CHG sites. This is very similar to the situation in the *OsSPL14*^{WFP} allele (Miura et al. 2010), but different from *afo*, in which the whole promoter region is hypermethylated (Wang et al. 2010).

In a previous study, we observed that the insertion of a rearranged retrotransposon into the intron of *OsMIK* caused diminished expression, probably also through increased methylation of a short fragment, particularly, of the two nucleotides at a putative transcription start site (Zhao et al. 2013). The significant increase of methylation in *Epi-GUN4* was also localized at the ARE in the promoter region, which together indicates that methylation of cytosines within specific elements can severely impact transcription, and thus downregulates the expression of the epi-allele.

Origin of the *epi-GUN4* allele

HYB was selected in the M_2 population of LTB, which was mutagenized by gamma rays (Zhou et al. 2006a). It is difficult to prove whether the *xantha* mutation was spontaneously generated or, as speculated, induced by gamma rays. However, *epi-GUN4* is not the first epi-allele reported in induced mutants. Indeed, a paramutation induced by the chemical mutagen ethyl methanesulfonate (EMS) was reported almost half a century ago (Axtell and Brink 1967). In Arabidopsis, two epigenetically controlled late flowering mutants were induced by either EMS (*fwa-1*) or fast neutrons (*fwa-2*) (Soppe et al. 2000). Therefore, it is possible that epigenetic variations can be induced by mutagenic treatment in plants.

In attempting to explain the generation of *fwa* epi-alleles induced by EMS, Soppe et al. (2000) proposed three possible causes, one of which was that the *FWA* hypomethylation was a direct consequence of the mutagenesis via disruption of overall genomic methylation. It is also possible that *epi-GUN4* was generated by gamma irradiation, because it has been shown that physical mutagenic treatments like spaceflight can induce both transient and heritable alterations in DNA methylation and gene expression in rice (*Oryza sativa* L.) (Ou et al. 2009). Artificial mutagenesis has become an important tool for plant breeding and functional genomics, but knowledge of the origin of induced genetic variations is currently very limited (Shu et al. 2012). The revelation of *epi-GUN4* and other epi-mutations previously reported suggests that epigenetic modification might be one of the causes of genetic variations; hence, this should be considered in an attempt to determine the causal gene of a mutated trait.

In summary, our study started with positional cloning of the gene underpinning the *xantha* marker trait and ended with the finding of *epi-GUN4* as the causal gene. This finding is expected to serve as a starting point for future research on *OsGUN4* function in Chl biosynthesis and regulation, as well as in retrograde regulation in response to oxidative stress in rice. In this regard, the new allelic *OsGUN4* mutants will be excellent genetic resources. This study also presents an interesting example of an epigenetically controlled trait of practical agronomic importance; because the extent to which epigenetic variation contributes to phenotypic variation in plants is still not well understood, such examples are certainly very valuable. Furthermore, this investigation quite naturally has raised a number of intriguing questions, e.g., what was the molecular biological process leading to the generation of the *epi-GUN4* allele? Why and how does cytosine methylation in the ARE inhibit transcription? Future studies may shed light on these fundamental aspects of research in epigenetics and retrograde regulation.

Author contributions QYS and JZH designed the research work. RQL, HJZ, HWF, YFL and GZL performed the experiments and analyzed the data together with QYS. RQL and JZH drafted the manuscript. QYS, JZH improved the manuscript.

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Conflict of interest The authors declare that they have no conflict of interest.

Ethical standard The authors declare that the experiments comply with the current laws of China.

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