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Characterization of the *NGP4A* Gene in Regulating Grain Number Per Panicle of Rice (*Oryza sativa* L.)

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Abstract: Grain number per panicle (GNPP) is a major factor influencing rice yield (*Oryza sativa* L.). However, the molecular mechanisms of GNPP determination are not well understood. A rice GNPP mutant, *ngp4a*, was isolated from an ethyl methanesulfonate-mutagenized rice library of *japonica* Nipponbare. *ngp4a* produced fewer grains than wild-type plants at maturity as the number of secondary branches decreased significantly. The mutant phenotype of *ngp4a* was controlled by a recessive nuclear gene, which was fine-mapped into a 155.2 kb region on chromosome 4. One GNPP-related gene, *Gnp4/LAX2* (*LOC_Os04g32510*), was found in the mapped region. The deletion of 3-bp nucleotides in the first exon of *NGP4A* resulted in a threonine residue loss. The mutation in *NGP4A* was responsible for the mutant phenotype of *ngp4a*. These results suggest that *NGP4A* is a new allele for *Gnp4* and *LAX2*, while the mutant phenotype and underlying causation differed. Notably, transcriptome analysis revealed that *NGP4A* could regulate GNPP determination through the phenylpropanoid biosynthesis and mitogen-activated protein kinase signaling pathways. Our results further elucidated the vital roles of *Gnp4/LAX2* in GNPP determination, providing a new genetic resource and theoretical basis to further explore the molecular mechanisms of GNPP in rice.

Keywords: rice; grain number per panicle; yield; allele; transcriptome sequencing

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1. Introduction

Rice (*Oryza sativa* L.) is one of the major food crops in the world, with nearly half of the world's population depending on rice as a staple food, particularly Southeast Asia [1]. Owing to the decreasing arable land area and increasing population, breeding rice cultivars with higher yields is crucial for food security [2]. Rice yield is mainly determined by three traits—number of panicles, grain number per panicle (GNPP), and grain weight—all of which are typical quantitative traits and are controlled by numerous genes [3]. Among these three traits, GNPP is a major contributor to rice yield and is crucial for high-yield rice breeding.

In rice breeding practice, different strategies for increasing rice yield have been proposed in China and abroad. For example, the International Rice Research Institute (IRRI) proposed a strategy of "fewer tillers and larger panicles [4]". Yuan et al. [5] proposed a super-high-yield ideal architectural feature of "high canopy layer, low panicle layer, large panicle, and high lodging resistance". Yang et al. [6] proposed a strategy of "upright and large panicle" for *japonica* rice breeding in northern China. Zhou et al. [7] proposed a strategy of "super high-yield breeding of inter-subspecies heavy panicle hybrid rice". While each of these high-yield breeding strategies has unique characteristics, they are all based on the principles of increasing the number of grains per panicle and increasing the grain weight per panicle, thereby increasing rice yield. Therefore, the development of large panicles with high GNPP has been gaining increasing attention in high-yield breeding programs for rice, and it is of great theoretical significance to clarify the regulatory mechanism of GNPP for the molecular design breeding of rice.

To this date, 238 quantitative trait loci (QTLs) controlling GNPP have been registered for rice on the Gramene website (http://www.gramene.org) (accessed on 2 January 2022). These QTLs are distributed across the 12 chromosomes of rice, and some of them have been well characterized [8,9]. Various mutants are valuable genetic resources for the functional analysis of genes and the elucidation of complex biological processes [10–12]. As excellent materials in such studies, GNPP mutants have been widely used to explore the molecular mechanisms of GNPP determination. For example, Zhao et al. [13] constructed a mutant library by mutating a rice variety YIL55 and investigated a mutant *pay1* with an upright and compact architecture. The overexpression of *PAY1* leads to fewer tillers, taller plant height, more secondary branches, a larger GNPP, and higher grain yield. *IPA1* encodes a squamosa promoter binding protein-like protein and positively regulates the number of branches and GNPP [13]. *MOC1* encodes a GRAS-family nuclear protein and positively regulates the tiller number, branch number, and GNPP in rice [14]. *Gnp4/LAX2* encodes a nuclear protein that regulates the formation of the axillary meristem and positively regulates the number of secondary branches and GNPP [15,16]. *Gnp4* and *LAX2* share the same locus in the long arm of rice chromosome 4. Moreover, numerous genes, such as *LAX1* [17], *SP1* [18], *DEP1* [19], *TAW1* [20], *OsNAC2* [21], *GNP1* [22], *DST* [23], and *PROG1* [24], have been reported to regulate GNPP. However, the molecular mechanisms by which these genes regulate the GNPP are far from being well understood, and some have not yet been cloned. Therefore, the regulation of GNPP warrants further investigation. It may be possible to increase the GNPP using related genes, thus increasing rice grain yield.

In this study, we identified a GNPP mutant *ngp4a* in rice. *ngp4a* was affected by a single recessive nuclear gene, which was named *NGP4A*. Using map-based cloning, *NGP4A* was fine mapped into a 155.2 kb region on chromosome 4. The results of the sequence alignment showed that 3 bp nucleotides were deleted in the first exon of *LOC_Os04g32510* in *ngp4a*, which resulted in a threonine residue loss. The complementation analysis confirmed that the genetic mutation of *LOC_Os04g32510* was responsible for the *ngp4a* mutant phenotype. Furthermore, we determined the global transcriptome profile of *ngp4a* to explore the major molecular function of *NGP4A*. This study laid a foundation to further illustrate the regulatory mechanism and application potential of *NGP4A* on GNPP in rice.

2. Materials and Methods

2.1. Plant Materials and Agronomic Evaluation

The *ngp4a* mutant was obtained from a mutant library, which was generated by subjecting Nipponbare (*O. sativa* L. ssp. *japonica*) to ethyl methanesulfonate (EMS). To map the *NGP4A* gene, an F_2 population was developed from the cross between *ngp4a* and a rice variety Minghui 63 (*O. sativa* L. ssp. *indica*). All plants were planted under natural conditions in a paddy field in Nanchang City, Jiangxi Province, China. Agronomic traits, including plant height, tiller number, main panicle length, primary branches of the main panicle, secondary branches of the main panicle, total spikelets of the main panicle, seed setting rate, 1000-grain weight, grain length, and grain width, were measured after maturity. The plant height was measured from the base of the plant to the top of the spike. The main panicle length was measured from the panicle neck to the panicle tip of the main panicle. The seed setting rate was calculated as the percentage of the filled grain number to the total grain number of the main panicle. The grain length and width were measured by an electronic digital vernier caliper, and fully filled grains were used for measuring the 1000-grain weight. The plant height, tiller number, and main panicle length were measured in the field. The remaining agronomic traits were measured in the laboratory after harvest. For each agronomic trait, 10 plants were evaluated as biological replicates. The average and SD values of the data were analyzed using Excel software. Statistical analysis was performed using the Student's *t*-test.

2.2. Primer Development and Gene Mapping

Simple sequence repeat (SSR) primers were obtained from the Gramene website (http://www.gramene.org/microsat/) (accessed on 2 January 2019) based on the SSR linkage map constructed by McCouch et al. [25]. Insertion/deletion (InDel) primers were developed based on the different sequences between Nipponbare and 9311 (*O. sativa ssp. indica*) and designed by the Primer3 (v.0.4.0) software (https://bioinfo.ut.ee/primer3-0.4.0/) (accessed on 2 January 2019). All primers were synthesized by Sangon Biotechnology (Sangon Tech, Shanghai, China) and conserved in the laboratory. To screen linked markers, two DNA pools were constructed by screening the GNPP phenotype in the F_2 population after maturity. The first pool was composed of 20 individual plants that showed the GNPP mutant phenotype of *ngp4a*, while the second pool was composed of 20 individual plants that showed the wild-type GNPP phenotype. In total, 326 SSR markers covering the 12 chromosomes of rice were screened by the two parents, *ngp4a* and Minghui 63, and the individuals from the F_2 population with the mutant GNPP phenotype were used for fine mapping with newly designed InDel markers. Primers used in this study are listed in Table S1.

2.3. Vector Construction and Plant Transformation

Based on the mapping results and literature references [15,16], *LOC_Os04g32510* was considered the most likely candidate gene in the gene mapping region. For the genetic complementation test, a DNA fragment containing the entire *LOC_Os04g32510* genomic sequence and 3000 bp upstream promoter sequence was amplified from Nipponbare using Q5 high-fidelity DNA polymerase (New England BioLabs, Hitchin, UK). Then, the purified and verified fragment was cloned into a pBWA(V)HII-CCDB-TNOS vector (Biorun biological technology Co., Ltd., Wuhan, China), which was digested by the ECO31I restriction enzyme to generate the resulting binary vector. Subsequently, the resulting binary vector was introduced into the *ngp4a* mutant using an *Agrobacterium tumefaciens*-mediated genetic transformation method as previously reported [26]. The PCR products were purified and sequenced by Shanghai Sangon Biological Engineering Technology and Services Co., Ltd. (Shanghai, China).

2.4. Transcriptome Sequencing

For transcriptome sequencing, *ngp4a* and wild-type plants were grown in hydroponic cultures using an IRRI liquid culture medium recipe (IRRI nutrient solution). At the four-leaf stage, the whole seedlings (all aerial organs plus root tissue) of *ngp4a* and wild-type were collected and immediately frozen in liquid nitrogen. To minimize the effect of transcriptome unevenness among plants, for RNA extraction, 10 *ngp4a* mutant and 10 wild-type whole seedlings were collected and mixed separately to form two libraries. Total RNA was then isolated from the samples using the Trizol reagent (Solarbio Science & Technology Co., Ltd., Beijing, China) following the manufacturer's instructions. RNA quality and quantity were checked using agarose gel electrophoresis and a Nanodrop 2000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). Subsequently, the qualified RNA samples were used for transcriptome sequencing. Transcriptome sequencing was performed by BioMarker Technology (Beijing, China) according to the manufacturer's recommendations.

3. Results

3.1. Characterization of ngp4a Mutant

To characterize the mutant traits of *ngp4a*, we compared the major agronomic traits between *ngp4a* and wild-type plants. The morphology of *ngp4a* from the seedling to the end of the vegetative stage was similar to that of the wild-type plants. However, *ngp4a* produced fewer grains than the wild-type plants at maturity. The quantitative analysis of the panicles revealed that the number of the primary branches was not affected significantly in the *ngp4a* mutant plants, while the number of secondary branches decreased significantly (Figure 1). On average, the wild-type plants had approximately 36.7 secondary branches

and 166.7 spikelets on the main panicle, while the mutant plants had approximately only 10.0 secondary branches and 106.3 spikelets on the main panicle, which suggests that the GNPP mutant appearance of *ngp4a* was due to a reduced number of secondary branches and total spikelets of the panicle. The grain size was also affected in the mutant. The statistical analysis revealed that the grain width was not affected in the mutant compared to the wild-type, while the grain length of the mutant increased significantly, which increased the 1000-grain weight. In addition, other major agronomic traits of *ngp4a*, including the plant height, main panicle length, and tiller number, decreased significantly compared with the wild-type plants (Table 1).

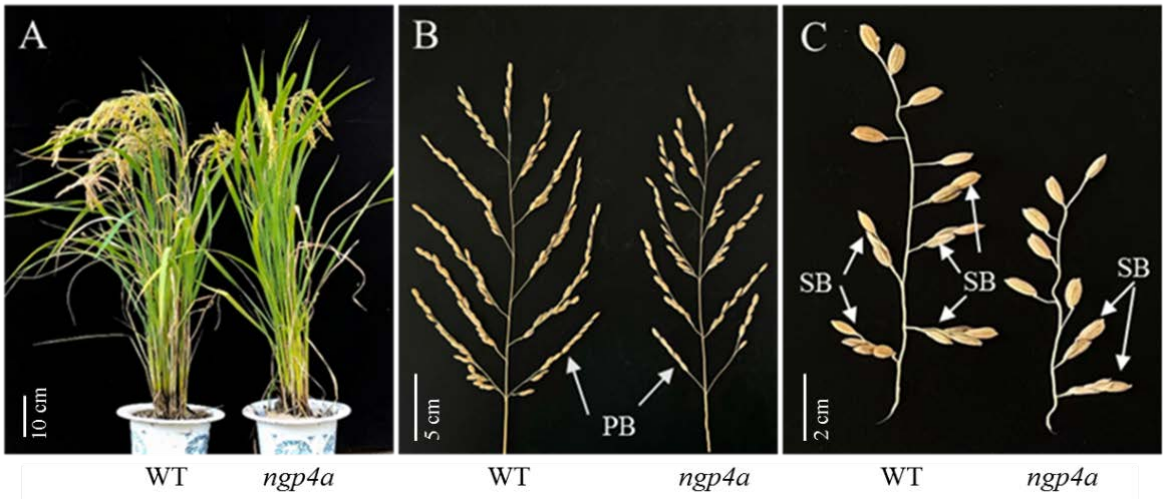

Figure 1. Phenotypic traits of *ngp4a* and wild-type plants. (**A**): Plant phenotypes of the *ngp4a* mutant at maturity. (**B**): Main panicle. (**C**): Primary and secondary branches. PB: primary branch. SB: secondary branch. *ngp4a*: mutant plants; WT: wild-type plants.

Table 1. Comparison of major agronomic traits between *ngp4a* and wild-type plants. *ngp4a*: mutant plants; WT: wild-type plants.

Traits	Wild Type (WT)	*ngp4a*	Compared with WT (%)
Plant height (cm)	95.7 ± 1.5	84.0 ± 0.8	−12.2 **
Tiller number	23.7 ± 1.5	16.0 ± 0.5	−32.5 **
Main panicle length (cm)	29.3 ± 1.1	26.9 ± 1.0	−8.2 *
Primary branches of main panicle	10.7 ± 0.6	10.0 ± 0.3	−6.5
Secondary branches of main panicle	36.7 ± 2.1	10.0 ± 0.5	−72.8 **
Total spikelets of main panicle	166.7 ± 4.7	106.3 ± 2.5	−36.2 **
Seed setting rate (%)	85.0 ± 1.4	88.01 ± 1.8	+3.5
1000-grain weight (g)	22.8 ± 0.4	26.09 ± 0.7	+14.4 *
Grain length (mm)	7.3 ± 0.1	8.0 ± 0.1	+9.6 *
Grain width (mm)	3.8 ± 0.1	3.9 ± 0.1	+2.6

Note: * represents a significant difference at the 0.05 level. ** represents a significant difference at the 0.01 level. For each agronomic trait, 10 plants as biological replicates were evaluated. All data are represented as mean ± SD. Statistical analysis was performed with the Student's *t*-test.

3.2. Fine Mapping of NGP4A

An F_2 population was developed from the cross between *ngp4a* and Minghui 63 for the genetic analysis of the *ngp4a* mutant. The phenotypic analysis revealed that the F_1 plants had the wild-type phenotype; among the 3632 F_2 individuals, 2682 plants had the wild-type GNPP phenotype and the rest had the mutant GNPP phenotype. The segregation ratio was approximately equal to 2.82:1 ($\chi^2 = 2.59$) and was in accordance with the expected ratio of 3:1, which suggests that the mutant phenotype of *ngp4a* was controlled by a single nuclear recessive gene, which was named *NGP4A*. To locate the *NGP4A* gene, we

performed map-based cloning. By screening 326 pairs of SSR markers scattered on the rice 12 chromosomes with proportional spacing, we found that 158 pairs of markers exhibited distinct polymorphisms between the two parents *ngp4a* and Minghui 63, and these markers were then used for analyzing the relationship with the *NGP4A* gene. Finally, we found four SSR markers (RM5687, RM6314, RM5424, and RM7563) on chromosome 4 that were linked with *NGP4A*. Subsequently, using 120 recessive individuals from the F_2 population, the region of *NGP4A* was preliminary framed between markers RM6314 and RM5424 with genetic distances of 6.3 and 5.0 cM, respectively. To further map *NGP4A*, we used 950 recessive individuals and developed new InDel markers in the preliminary mapping region. Finally, *NGP4A* was narrowed down to a 0.9 cM interval between InDel4-44 and InDel4-59, and the physical distance was approximately 155.2 kb (Figure 2B,C).

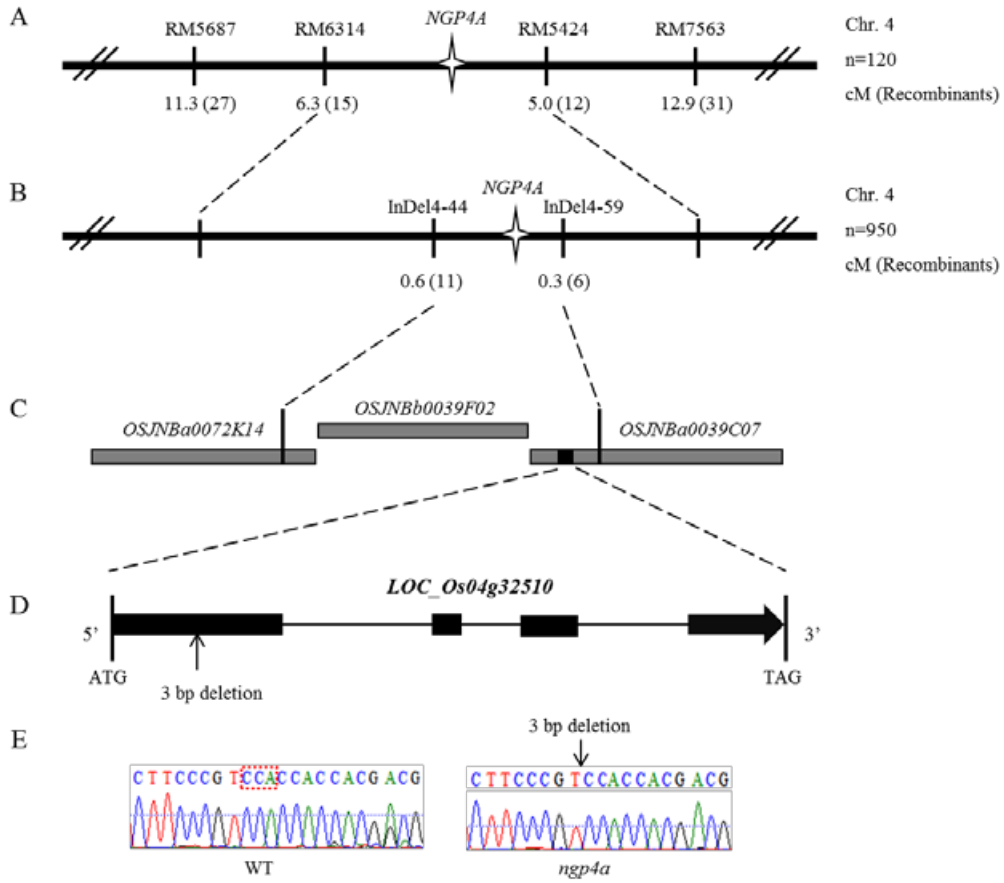

Figure 2. Genetic and physical map of *NGP4A*. (**A**): *NGP4A* locus mapped in the long arm of chromosome 4. (**B**,**C**): Fine mapping of *NGP4A*. The numbers in the parentheses indicate the number of recombinants. (**D**): Structure of *NGP4A*. The black boxes indicate the coding sequence and the lines between the boxes represent introns. (**E**): Mutant site of *NGP4A*. The 3 bp nucleotide deletion of *NGP4A* is framed by the red dotted box. WT: wild-type plants.

3.3. Sequence and Complementation Analysis

Ten genes were predicted according to the annotation in the Rice Genome Annotation Project (http://rice.uga.edu/) (accessed on 15 February 2019) within the fine mapped region. Among these genes, one (*LOC_Os04g32510*) was involved in the regulation of the GNPP of rice. To clarify this further, we amplified the entire genomic sequence of *LOC_Os04g32510* from the *ngp4a* mutant and its wild-type parent Nipponbare. Specific primers were designed according to the genome sequence of Nipponbare. The primers for gene cloning and sequence analysis of *LOC_Os04g32510* are listed in Table S1. The results of the sequence alignment showed that 3 bp nucleotides were deleted in the first exon of *LOC_Os04g32510* in *ngp4a*, which resulted in the loss of a threonine residue (Figure 2D,E).

Therefore, we speculated that the genetic variation of *LOC_Os04g32510* could be the underlying reason for the mutant phenotype of *ngp4a*. To confirm the defect of *LOC_Os04g32510* as the causal mutation of *ngp4a*, a DNA segment including the entire genomic sequence of *LOC_Os04g32510* and its promoter was introduced into *ngp4a* with *A. tumefaciens*-mediated transformation. Twelve positive transgenic lines in the T_2 generation were obtained in total, all of which had a wild-type panicle phenotype (Figure 3). Meanwhile, no significant differences were observed between the wild-type and complementation transgenic plants in terms of other major agronomic traits (Table 2). These results demonstrated that the genetic variation of *LOC_Os04g32510* was responsible for the *ngp4a* mutant phenotype, and *LOC_Os04g32510* was indeed the *NGP4A* gene.

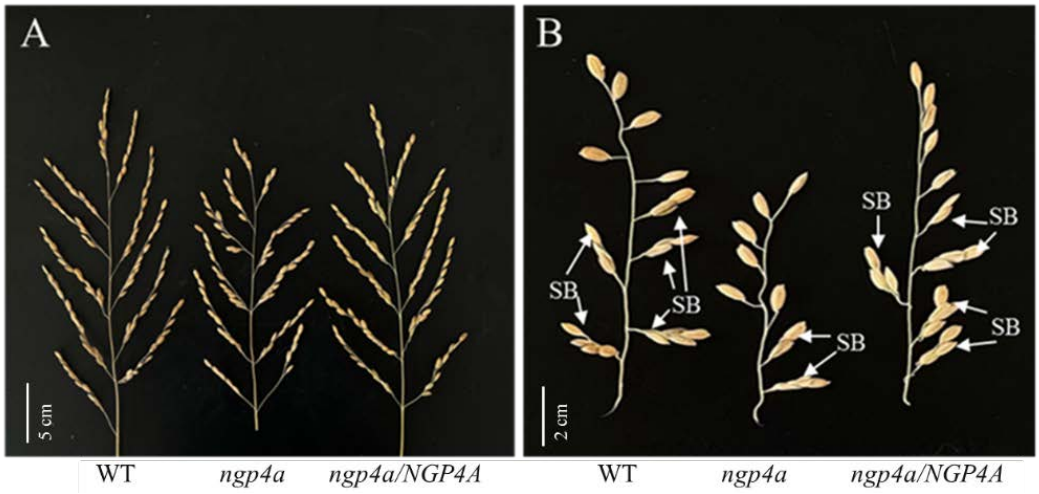

Figure 3. Panicle phenotype of complementation transgenic plants. While *ngp4a* exhibited the grain number per panicle mutant phenotype, the complementation transgenic plants exhibited the wild-type phenotype. (**A**): Main panicle. (**B**): Primary and secondary branches. WT: Wild type; *ngp4a*: mutant; *ngp4a/NGP4A*: complementation transgenic plants. SB: Secondary branch.

Table 2. Comparison of major agronomic traits between complementation transgenic plants *ngp4a/NGP4A* and wild-type plants.

Traits	Wild Type (WT)	*ngp4a/ NGP4A*	Compared with WT (%)
Plant height (cm)	95.6 ± 0.8	93.0 ± 1.1	−2.7
Tiller number	22.7 ± 1.5	23.3 ± 0.9	+2.8
Main panicle length (cm)	30.0 ± 0.4	28.8 ± 0.7	−4.0
Primary branches of main panicle	10.7 ± 0.6	10.3 ± 0.6	−3.1
Secondary branches of main panicle	35.7 ± 1.7	33.8 ± 2.5	−5.2
Total spikelets of main panicle	172.0 ± 5.3	162.7 ± 3.7	−5.4
Seed setting rate (%)	85.5 ± 0.7	87.2 ± 0.5	+2.0
1000-grain weight (g)	22.8 ± 0.9	23.4 ± 0.8	+2.8
Grain length (mm)	7.3 ± 0.1	7.4 ± 0.1	+0.5
Grain width (mm)	3.8 ± 0.1	3.9 ± 0.1	+0.9

3.4. Transcriptome Analysis

To explore the possible mechanisms underlying the *ngp4a* mutant phenotype, we performed transcriptome sequencing for *ngp4a* and wild-type Nipponbare. After filtering, the total numbers of clean reads were 20.48 and 20.59 million for *ngp4a* and wild-type plants, respectively. At least 93.53% of the clean reads had a quality score of Q30 for both types of samples. The GC contents were 53.10% and 52.83% for *ngp4a* and wild-type plants, respectively. In total, 39,516,745 (96.47%) and 39,836,670 (96.72%) clean reads from *ngp4a* and wild-type plants, respectively, were successfully and uniquely mapped to the rice

genome using the HISAT2 software (Table 3). These results indicated the high quality of the sequencing data and were competent for subsequent analysis.

Table 3. RNA sequencing data of two samples.

Sample	Wild Type (WT)	*ngp4a*
Clean reads	20,593,555	20,481,813
GC content	52.83%	53.10%
Clean bases	6,150,165,034	6,126,925,284
Total mapped reads (%)	39,836,670 (96.72%)	39,516,745 (96.47%)
Multiple mapped reads (%)	1,184,323 (2.88%)	1,130,576 (2.76%)
Unique mapped reads (%)	38,652,348 (93.85%)	38,386,169 (93.71%)

The expression quantities of 30,825 and 30,625 genes were detected in *ngp4a* and wild-type plants, respectively. Among them, 784 genes were identified as differentially expressed genes (DEGs) with the cut-off for the false discovery rate of <0.01 and fold change of $\geq$2 (Table S2). Of these DEGs, 581 were up-regulated and the rest were significantly down-regulated. These DEGs were distributed across all chromosomes but most were located on chromosome 1. To characterize the function of DEGs, Gene Ontology (GO) term enrichment analysis was performed to detect the functional pathways of the mutant traits. In total, 538 DEGs were assigned into three GO categories. The statistical results demonstrated that most of the DEGs were related to the cellular component, followed by the biological process and molecular function categories (Figure 4). Many DEGs associated with metabolic process, cellular process, and single-organism process GO terms were significantly enriched, thereby suggesting that extensive biological processes were altered in the mutant plants. Within the cellular component category, cell, cell part, and membrane GO terms were significantly enriched, thereby suggesting that *NGP4A* affected the membrane and cell parts in the mutant plants. Many DEGs associated with binding, catalytic activity, and transporter activity were also significantly enriched, thereby suggesting that *NGP4A* influenced the molecular function of the mutants. Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis was undertaken to predict the biochemical pathways associated with the DEGs (Figure 5). Interestingly, 229 DEGs were assigned to 49 KEGG pathways, of which six pathways were significantly enriched (q-value $\leq$ 0.05) (Table S3). Among the KEGG pathway-annotated DEGs, 39 were associated with plant–pathogen interaction, 28 were associated with plant hormone signal transduction, and 25 were associated with the mitogen-activated protein kinase (MAPK) signaling pathway-plant. These were the three largest groups of DEGs according to KEGG pathway analysis. However, the *NGP4A* mutation had the largest impact on the phenylpropanoid biosynthesis (q-value = 0.00115) and MAPK signaling pathways (q-value = 0.00125).

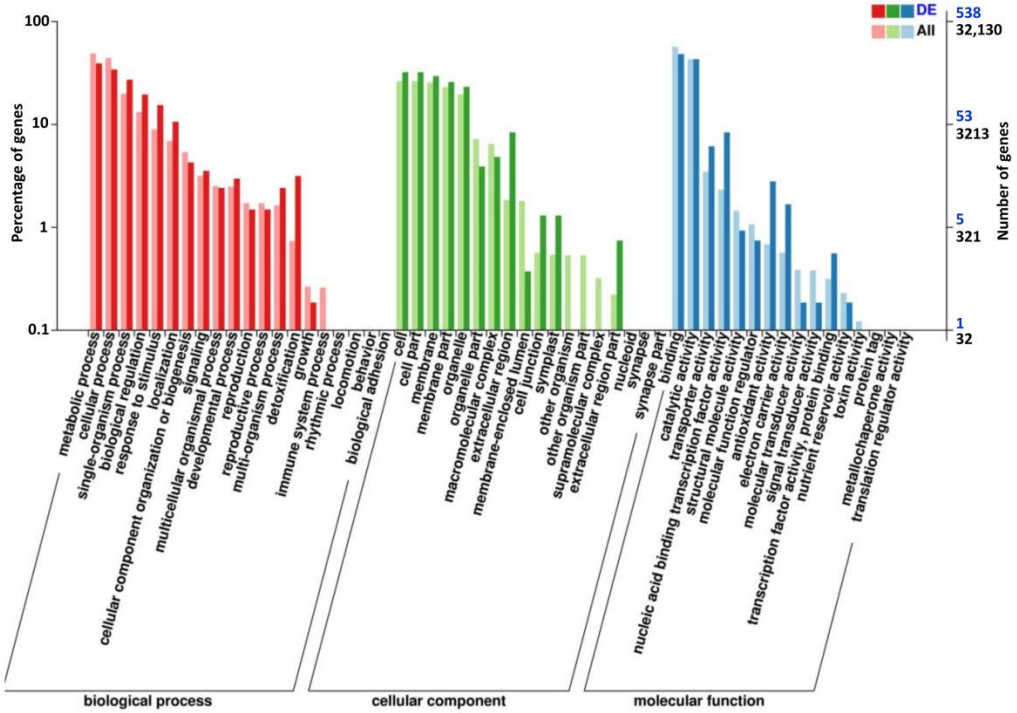

Figure 4. Gene ontology (GO) classification of the differentially expressed genes.

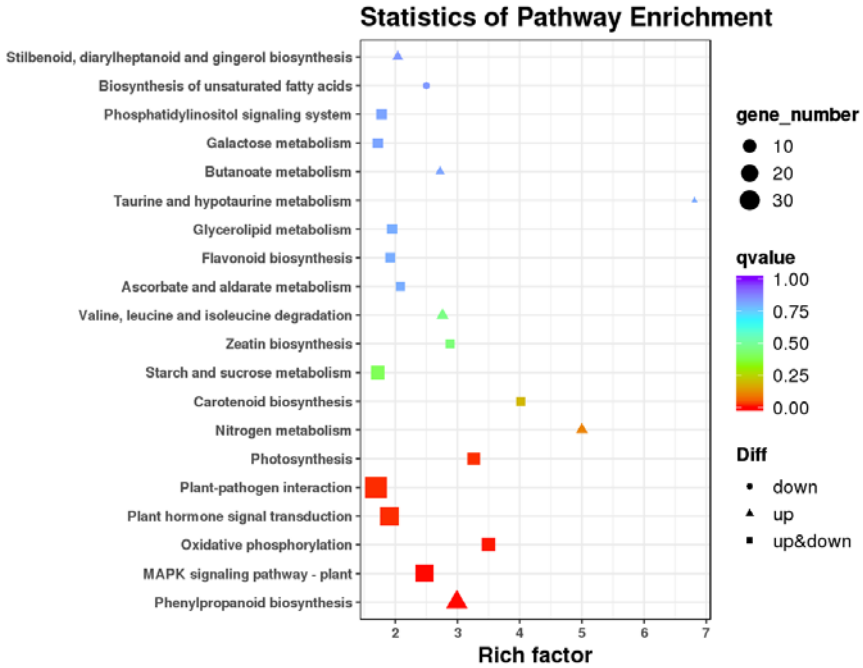

Figure 5. Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway assignments for the differentially expressed genes.

4. Discussion

The development of high-yield cultivars is one of the most important goals of rice breeding. GNPP plays a decisive role in rice yield, and extensive attention has been paid to improving the GNPP trait in various plants [27,28]. Nevertheless, the GNPP mechanism is still poorly understood, especially in rice. Mutant analysis is a powerful approach to illuminate the underlying molecular mechanisms in the complex biological processes of GNPP.

In this study, a GNPP mutant, *ngp4a*, was found in the mutant library. The GNPP of *ngp4a* was significantly reduced compared with that of wild-type plants. Meanwhile, the other major agronomic traits, including plant height, tiller number, panicle length, grain length, and 1000-grain weight, were significantly changed in the mutant plants, indicating that *NGP4A* had pleiotropic effects, thereby corroborating the results of previously published studies. For example, *GNS4* encodes a cytochrome P450 protein, and *GNS4*-overexpressing rice plants have significantly increased grain weight and GNPP [29]. *GW2* encodes a ring-type protein with E3 ubiquitin ligase activity, whose loss of function results in a significant increase in 1000-grain weight and a decrease in GNPP [30]. *GN2* encodes a peptide of 70 amino acids, and *GN2*-overexpressing plants exhibit reduced grain numbers, plant height, and heading stage [31]. The results of these studies suggest that a single gene can simultaneously affect several phenotypic traits.

Using fine mapping, *NGP4A* was finally delimited into a 155.2 kb region on chromosome 4. Based on the annotation results of the Rice Genome Annotation Project (http://rice.uga.edu/) (accessed on 15 February 2019), ten genes were predicted in the fine-mapped region. Among these genes, one (*LOC_Os04g32510*) was named *Gnp4* and *LAX2* and is involved in the regulation of the GNPP trait of rice [15,16]. To this end, *LOC_Os04g32510* was considered the most likely candidate gene in the gene mapping region. Using sequence and complementation analysis, we confirmed that *NGP4A* was a novel allele for *Gnp4* and *LAX2*. However, *ngp4a* was different from *gnp4* and *lax2* in the mutant phenotype and underlying causation. For *gnp4*, there was no significant difference in the primary branch number and grain width, while the number of secondary branches and grains decreased significantly, and the grain length and 1000-grain weight increased significantly; these results were similar to those of *ngp4a*. However, no significant difference was observed in the tillering number of *gnp4*, while that of *ngp4a* decreased significantly. Meanwhile, the number of secondary branches, grains, and tillers of *lax2* decreased significantly; these results were similar to those for *ngp4a*. The number of primary branches of *lax2* increased; however, no significant difference was obtained in the number of primary branches of *ngp4a*. These results showed that different mutant phenotypes occurred in the three mutants, *ngp4a*, *gnp4*, and *lax2*. Furthermore, 3 bp nucleotides were deleted in the first exon of *LOC_Os04g32510* in *ngp4a*, which resulted in the loss of a threonine residue. However, no nucleotide differences occurred in *gnp4* and the phenotypic mutation could have been caused by the changes in DNA methylation levels in the promoter of *LOC_Os04g32510*. Tabuchi et al. [16] isolated three independent mutant alleles for *LAX2* (*LAX2-1*, *LAX2-2*, and *LAX2-3*). They found that *lax2-1* and *lax2-3* deleted a nucleotide in the first and third exons of *LOC_Os04g32510*, respectively, while a nucleotide was inserted into the first exon of *LOC_Os04g32510* in *lax2-2*. These results suggested that the mutation pattern of *NGP4A* was different from those of *Gnp4* and *LAX2*.

The molecular mechanisms through which *Gnp4* and *LAX2* affect GNPP are poorly understood, warranting further investigation. *MOC1* [14] and *LAX1* [16] are two important genes in axillary meristem formation. Tabuchi et al. [16] proposed a regulation model for *LAX2* and suggested that there were multiple pathways in which *LAX1*, *LAX2*, and *MOC1* function for branching. *LAX2* acts as a cofactor of *LAX1*, and it is unlikely that *LAX2* regulates *LAX1* expression. Meanwhile, *LAX2* may or may not form dimers with *MOC1*. Moreover, Zhang et al. [15] reported that *Gnp4* and *LAX1* indirectly regulate tiller formation and directly or indirectly regulate secondary branch formation in multiple genetic pathways. Similar results were obtained in our transcriptome analysis as the expression levels of *LAX1* and *MOC1* did not change significantly in *ngp4a* mutant plants.

In recent years, the rapid development of high-throughput sequencing technology has resulted in the emergence of transcriptome analysis by RNA sequencing as a powerful strategy to explore molecular mechanisms underlying complex biological processes [32,33]. In this study, we found that the *NGP4A* mutation caused global changes in the rice transcriptome using RNA sequencing. Six significantly enriched pathways were observed in *ngp4a*, including phenylpropanoid biosynthesis, MAPK signaling, oxidative phosphorylation,

plant hormone signal transduction, plant–pathogen interaction, and photosynthesis. The *NGP4A* mutation had the largest impact on the phenylpropanoid biosynthesis and MAPK signaling pathways. The phenylpropanoid biosynthesis pathway, identified to synthesize lignin, anthocyanins, and flavonoids in plants, is important for plant growth and development and contributes to plant responses to abiotic and biotic stresses [34–37]. Recent studies have suggested that the seed size of plants is correlated with lignin content. For example, the overexpression of *SlMBP3* results in a 60% increase in lignin content and larger seeds in tomato [38]. *GS3.1* regulates grain size via metabolic flux allocation between two branches of phenylpropanoid metabolism in rice [39]. In the present study, the grain length of *ngp4a* was significantly affected, which implies that the phenylpropanoid biosynthesis pathway may play multiple roles in the phenotypic mutation of grain number and seed size. MAPK is a serine/threonine protein kinase, whose cascades are highly conserved and is associated with ubiquitous signaling pathways in eukaryotes [40,41]. Plant MAPK signaling modules play fundamental roles in many aspects of biological processes, including immunity, stress responses, and developmental programs [42,43]. To date, several genes involved in the MAPK signaling pathway associated with grain number have been identified and characterized in rice. Guo et al. [44] reported that *GSN1* can act as a negative regulator of a MAPK cascade that determines panicle architecture. Compared with wild-type plants, the grain length, grain width, and 1000-grain weight of the *gsn1* mutant increased significantly, while the GNPP and seed setting rate decreased significantly. Other examples include an MAPK gene *OsMPK15*, which results in a marked decrease in the GNPP and seed setting rate and an increase in the 1000-grain weight in mutant plants [45]. *OsER1* can act upstream of a MAPK cascade to control the spikelet number of panicles [44]. Mutant *oser1* results in a markedly increased number of spikelets per panicle compared with the wild-type plants. Therefore, the up-regulation and down-regulation of core components of the MAPK signaling pathway in *ngp4a* suggest its flexibility in regulating the GNPP of rice, although the exact mechanisms remain unclear.

Furthermore, plant hormones, including cytokinin (CK), auxin, gibberellin (GA), abscisic acid (ABA), and brassinolactone (BR), involved in regulating the GNPP of rice, are becoming increasingly well understood [46–49]. A complex network of multiple hormonal pathways collide and communicate to regulate GNPP in rice. For example, *GNP1* is involved in GA biosynthesis and affects the GNPP in rice [22]. *Gn1a* is a gene for CK oxidase/dehydrogenase and reduces the expression of *Gn1a* that causes CK accumulation and increases the number of grains, thereby resulting in enhanced grain yield [23,50]. *OsRLCK57* negatively regulates the BR signaling pathway and reduces the expression of *OsRLCK57*, thereby resulting in significantly fewer tillers and panicle secondary branching [51]. Based on our transcriptome sequencing data, we found 28 DEGs that were significantly enriched in the pathways related to plant hormone signal transduction processes in *ngp4a* mutant plants. These findings suggest that the knockout of *NGP4A* altered plant hormone homeostasis, which may be the main cause of the GNPP mutant phenotype.

5. Conclusions

In conclusion, we characterized a novel GNPP mutant *ngp4a*. The genetic analysis and map-based cloning revealed that the causal gene *NGP4A* was a novel mutant allele gene for *Gnp4* and *LAX2*. However, *ngp4a* was different from *gnp4* and *lax2* in the mutant phenotype and underlying causation. The transcriptome analysis showed significant changes in the expression level of genes involved in phenylpropanoid biosynthesis, MAPK signaling, oxidative phosphorylation, plant hormone signal transduction, plant–pathogen interaction, and photosynthesis in *ngp4a.* These findings provide a theoretical basis for further molecular studies of *NGP4A* involved in the GNPP of rice and subsequent high-yield rice breeding programs.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agronomy12071549/s1, Table S1. Primers for map-based cloning

and genetic complementation of *NGP4A*. Table S2. Differentially expressed genes between *ngp4a* and wild-type plants. Table S3. KEGG pathways of the differentially expressed genes.

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