Genome-Wide Analysis Identified a Set of Conserved IncRNAs Associated with Domestication-Related Traits in Rice

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Abstract: Crop domestication, which gives rise to a number of desirable agronomic traits, represents a typical model system of plant evolution. Numerous genomic evidence has proven that noncoding RNAs such as microRNAs and phasiRNAs, as well as protein-coding genes, are selected during crop domestication. However, limited data shows plant long noncoding RNAs (lncRNAs) are also involved in this biological process. In this study, we performed strand-specific RNA sequencing of cultivated rice Oryza sativa ssp. japonica and O. sativa ssp. indica, and their wild progenitor O. rufipogon. We identified a total of 8528 IncRNAs, including 4072 IncRNAs in O. rufipogon, 2091 IncRNAs in japonica rice, and 2365 IncRNAs in indica rice. The IncRNAs expressed in wild rice were revealed to be shorter in length and had fewer exon numbers when compared with IncRNAs from cultivated rice. We also identified a number of conserved IncRNAs in the wild and cultivated rice. The functional study demonstrated that several of these conserved IncRNAs are associated with domestication-related traits in rice. Our findings revealed the feature and conservation of IncRNAs during rice domestication and will further promote functional studies of IncRNAs in rice.

Keywords: agronomic traits; conservation; crop domestication; cultivated rice; IncRNAs; wild rice

1. Introduction

The Oryza genus is an ideal model for studying mechanistic insights into crop domestication. The Asian cultivated rice Oryza sativa is one of the most important staple crops worldwide and was domesticated from its wild progenitor Oryza rufipogon ~10,000 years ago [1]. As cultivating high-yield rice varieties is the core objective in ancient breeding, a number of morphological and physiological changes appeared during the domestication process, especially the yield-related traits including panicle complexity and grain size [1–4]. For example, O. rufipogon and O. sativa exhibit strikingly different panicle architectures, from a low branching complexity and small grain size in O. rufipogon to a more complex panicle and larger seeds in O. sativa [5–7]. A similar divergence of domestication-related traits was also observed between the African rice O. glaberrima and its wild ancestor O. barthii [8].

Previous studies have reported that the two Asian rice varieties, O. sativa ssp. japonica and O. sativa ssp. indica, were domesticated from different O. rufipogon populations by two independent domestication events [9]. With the rapid advance of genome or tran-
over the past decades have provided important information on protein-degenerated awn, hull color, and pericarp cation, including increased seed sativa O. rufipogon investigated the panicle architectures, seed traits by manipulating the expression of lncRNAs. during rice domestication and demonstrated the potential to improve plant agronomic traits. Our functional study demonstrated that many conserved lncRNAs are associated with agro-numbers when compared with lncRNAs from cultivated rice. Comparative analysis and lncRNAs expressed in wi sativa genome biological roles for agronomic t traits changes during crop domestication, we performed a genome-wide analysis of IncRNAs from the seedlings and panicles of cultivated rice O. sativa ssp. japonica and O. sativa ssp. indica and the wild rice O. rufipogon. A number of IncRNAs expressed in wild rice were revealed to be shorter in length and have fewer exon numbers when compared with IncRNAs from cultivated rice. Comparative analysis and functional study demonstrated that many conserved IncRNAs are associated with agronomic traits in rice. Our results revealed the characteristics and conservation of IncRNAs during rice domestication and demonstrated the potential to improve plant agronomic traits by manipulating the expression of IncRNAs.

2. Results

2.1. Transcriptome-Wide Analysis of IncRNAs in Wild Rice and Cultivated Rice

To show vivid changes of the agronomic traits during rice domestication, we investigated the panicle architectures, seed-setting rate, and grain shape of three Oryza species: O. rufipogon collected from Hainan province, O. sativa ssp. japonica Nipponbare, and O. sativa ssp. indica 93-11. We found a series of profound changes appeared during domestication, including increased seed-setting rate, grain size, and grain weight, as well as a degenerated awn, hull color, and pericarp color (Supplementary Figure S1). Besides this, studies over the past decades have provided important information on protein-coding
genes in regulating domestication traits, such as shattering, plant architecture, heading date, tiller angle, stress adaptation, and grain quality. In this study, we asked whether long noncoding RNAs could also be involved in the rice domestication process.

To answer this question, we first collected samples of panicles and seedlings from the wild rice *Oryza rufipogon*, and two cultivated rice, *O. sativa* ssp. *japonica* and *indica*. We performed strand-specific paired-end deep sequencing to characterize lncRNAs and obtained 83.56 Gb clean reads in these six datasets with 89.18% of them able to be mapped to the referenced genomes of wild and cultivated rice [23–27] (Table 1). An average of 76.64% of these reads from six datasets were uniquely mapped to the *Oryza* genomes, followed by transcript assembly using the StringTie tool and obtained a total of 45,579 (including 26,657 mRNAs), 40,097 (including 31,313 mRNAs), and 41,149 (including 28,686 mRNAs) transcripts in *O. rufipogon*, *japonica* and *indica* groups, respectively. Basic filter processes and potential coding capability screening were than performed to exclude protein-coding genes, small noncoding RNAs that were shorter than 200 nt, and transcripts with FPKM (fragment per kilobase of transcript per million mapped reads) less than 0.1 (Figure 1a). We finally obtained 8528 lncRNAs from the six datasets, including 4072 lncRNAs in *O. rufipogon*, 2091 lncRNAs in *japonica* and 2365 lncRNAs in *indica* (Figure 1b, Supplementary Table S1–S3). In order to estimate whether these lncRNAs are novel or not, we aligned them with annotated lncRNAs in the reference genome database and previous literatures [21,28] and found more than a half of lncRNAs from *japonica* (1099 out of 2091, 52.56%) were known (Figure 1b and Supplementary Table S2), while none of the 4072 wild rice-derived lncRNAs and 2365 *indica* rice-derived lncRNAs were annotated before (Figure 1b, Supplementary Table S1 and S3).

Table 1. A summary of data from lncRNA sequencing.

| Index                  | *O. rufipogon* | *O. sativa* ssp. *japonica* | *O. sativa* ssp. *indica* |
|------------------------|----------------|-----------------------------|---------------------------|
|                       | Panicle        | Seedling                    | Panicle                    | Seedling                    |
| Raw reads              | 82,586,298     | 90,574,892                  | 88,408,434                 | 97,907,538                  | 93,570,604                  | 104,287,794                 |
| Clean reads            | 82,578,958     | 90,569,210                  | 88,402,696                 | 97,902,546                  | 93,564,262                  | 104,279,248                 |
| Clean bases            | 12.38 G        | 13.58 G                     | 13.25 G                    | 14.68 G                     | 14.03 G                     | 15.64 G                     |
| GC content             | 51.09%         | 46.50%                      | 49.66%                     | 48.10%                      | 49.67%                      | 48.91%                      |
| Total mapped reads (%) | 62,020,407     | 75,227,980                  | 80,629,086                 | 91,211,211                  | 81,866,743                  | 97,171,107                  |
| Unique mapped reads (%)| 61,024,157     | 73,364,876                  | 67,553,409                 | 60,706,447                  | 74,933,904                  | 78,992,468                  |
| Multiple mapped reads (%)| 996,250       | 1,863,104                   | 13,075,677                 | 30,504,764                  | 6,932,839                   | 18,178,639                  |
| Reads map to ‘exon’ (%)| (1.29%)        | (2.07%)                     | (15.06%)                   | (31.46%)                    | (7.55%)                     | (17.81%)                    |
| Reads map to ‘intron’ (%)| (53.04%)      | (40.58%)                    | (43.08%)                   | (19.42%)                    | (38.51%)                    | (31.60%)                    |
| Reads map to ‘intergenic’ (%)| (13.99%)   | (8.18%)                     | (6.51%)                    | (2.28%)                     | (8.21%)                     | (2.24%)                     |
| Read map to ‘exon’ (%)| (32.97%)       | (51.25%)                    | (50.41%)                   | (78.30%)                    | (53.28%)                    | (66.16%)                    |
Figure 1. Identification of lncRNAs from wild and cultivated rice. (a) Pipeline used to identify lncRNA transcripts in this study. (b) Comparison of lncRNAs identified in this study from previously annotated rice lncRNAs. The red box represents the number of novel lncRNAs identified in this study, and the black box indicates the number of previously annotated lncRNAs in the literature[21,28] that are included in our datasets.

2.2. Characteristics Comparison of lncRNAs in Wild and Cultivated Rice

Since most of the lncRNAs identified in this study were novel, we asked whether they had distinctive features between wild and cultivated rice. We performed t statistics for the following comparison between wild and cultivated rice and found lncRNAs in wild rice appeared to be shorter than that of cultivated rice, with the median length of 370 nt for wild rice-derived lncRNAs, while the median length of lncRNAs in japonica and indica rice were 670 nt (p < 0.0001) and 508 nt (p < 0.0001), respectively (Figure 2a). The wild rice-derived lncRNAs had fewer exons than that of cultivated rice, with 1.61 exons on average in wild rice versus 2.37 (p < 0.0001) and 1.76 (p < 0.0001) exons in japonica and indica, respectively. The percentage of single-exon lncRNAs in wild rice is 65.83%, and the percentage in japonica and indica rice were 50.39% and 61.96%, respectively (Figure 2b).

We also explored the characteristics of mRNAs in our datasets. Distinct from lncRNAs, the median mRNA length of protein-coding genes in wild rice was 1178 nt, which was lower than in japonica rice (1425 nt, p < 0.0001), but higher than in indica rice (912 nt, p < 0.0001) (Figure 2c). Additionally, the average exon number of mRNAs in wild rice were higher than in cultivated rice (O. rufipogon 5.85 vs. japonica rice 4.55, p < 0.0001; O. rufipogon 5.85 vs. indica rice 4.18, p < 0.0001) (Figure 2d), which was also distinct from the tendencies of lncRNAs. These observations suggested that the domestication process was accompanied by the increasing length and exon number of lncRNAs.
It is reported that a large number of lncRNAs are specifically expressed and involved in the sexual reproduction of rice. We next investigated the expression of lncRNAs in wild and cultivated rice. The results showed that lncRNAs were significantly higher expressed in panicles than in seedlings all through the three *Oryza* genomes. Statistical analysis suggested that the median log10(FPKM+1) value was 0.52 in panicles versus 0.06 in seedlings in wild rice (*p* < 0.0001), while the median values were 0.62 in panicles versus 0.44 in seedlings in *japonica* rice (*p* < 0.0001) and 0.66 in panicles versus 0.26 in seedlings in *indica* rice (*p* < 0.0001) (Figure 3a). A heatmap based on the value of log10(FPKM+1) was also generated to depict the difference in expression level among samples in each lncRNA, from which we could more clearly understand the generally higher expression of lncRNAs in panicles than in seedlings (Figure 3b). Among the 8528 lncRNAs identified in this study, we found a large proportion of lncRNAs were differentially expressed in panicles and seedlings (fold change > 2, and FDR < 0.05) (Supplementary Tables S1–S3), including 3040 out of 4072 lncRNAs (74.66%) in wild rice, 1104 out of 2091 lncRNAs (52.80%) in *japonica* rice, and 1659 out of 2365 lncRNAs (70.15%) in *indica* rice. To confirm the reliability of our datasets for lncRNA expression profiles, we experimentally validated the expression of twelve lncRNAs from wild and cultivated rice with different expression patterns. The results of qPCR analysis revealed that the expression of lncRNAs were consistent with those obtained from lncRNA sequencing (Supplementary Figure S2).
2.3. Conservation Analysis of IncRNAs That may be Associated with Rice Domestication

It has been well-characterized that IncRNAs are highly diverged at the nucleotide level among plant species but have high sequence conservation at the intra-species and sub-species levels [22]. We want to know whether IncRNA sequences are conserved or not between rice and its wild ancestor. After the sequence alignment of these 8528 IncRNAs identified in our datasets, we screened only 628 out of 4072 (15.42%) wild rice-expressed IncRNAs with homologous regions with 272 out of 2091 (13.01%) IncRNAs from *japonica* and 246 out of 2365 (10.40%) IncRNAs from *indica*, showing that only a few IncRNAs were sequence-conserved between wild and cultivated rice (Supplementary Table S4).

**Figure 3.** Expression analysis of IncRNAs from wild and cultivated rice. (a) The FPKM distribution of IncRNAs in panicle and seedling of wild and cultivated rice. (b) Heatmap of differentially expressed IncRNAs in panicle and seedling of wild and cultivated rice. All expression levels are normalized and displayed as log10(FPKM+1). The color from blue to red represents lowest to highest IncRNA expression.
The numbers of conserved IncRNAs with homologous sequences in wild and cultivated rice are not identical, implying that duplication or deletion of IncRNA copies occurred during rice domestication. We then performed an in-depth analysis of these sequence-conserved IncRNAs and a total of 633 matches were constructed among the 628/272/246 IncRNAs in wild/japonica/indica rice (Figure 4a and Supplementary Table S4). We found multiple IncRNAs in wild rice are homologous to a few of IncRNAs in cultivated rice (Figure 4a,b). MSTRG.10936.1 and MSTRG.4996.1 from O. sativa japonica are two examples that are sequence-conserved with 2 and 3 IncRNAs in indica but with 41 and 21 IncRNAs in O. rufipogon, respectively (Figure 4b). Sequence analyses of these two multiple-matched IncRNAs revealed that they were highly identical to two transposable elements, i.e., terminal-repeat retrotransposon in miniature ZO3 (GenBank accession: EF555578.1, identity: 96.50%) and transposon CACTG element RIM2-M337 (GenBank accession: BK000948.1, identity: 96.39%). These TE-associated IncRNAs are widely expressed in the wild rice, but only a few of them are detected in the cultivated rice, suggesting most of these TE-associated IncRNAs are silenced or excluded during the domestication of rice.

Next, we investigated the gene synteny information to assess the orthologous relationships of IncRNAs (Figure 4c and Supplementary Table S5). By analyzing the collinear segments of IncRNAs, we identified 78 IncRNA-matches comprised of 69 wild rice-derived IncRNAs and 55 japonica rice-derived IncRNAs. In the wild-indica rice group, 123 IncRNA-matches comprised of 105 wild rice-derived IncRNAs and 111 indica rice-derived IncRNAs were also determined. Only 60 IncRNA-matches comprised of 47 japonica rice-derived IncRNAs and 52 indica rice-derived IncRNAs showed positional-conservation in the japonica-indica rice group. A comprehensive comparison of IncRNA synteny between wild and cultivated rice suggested eight IncRNA-matches were positionally conserved in all the three Oryza genomes, indicating potential conserved roles of these IncRNAs during rice domestication (Figure 4c). Additionally, these results also showed that the conserved IncRNA synteny in wild-indica rice was stronger than in wild-japonica rice, providing evidence from the noncoding RNA perspective to support the latest hypothesis of indica rice originated from cross-hybridized ancient japonica cultivars and wild rice [9].

Figure 4. Circos plots of sequence conserved and positionally conserved IncRNAs that may be associated with rice domestication. (a) Distribution of sequence-conserved IncRNAs between O. rufipogon, japonica and indica. The 12 chromosomes in each set are shown in the outermost circle. Red lines in the second circle indicate the density of IncRNAs detected in this study. Heatmaps in the third and fourth circles represent the expression level of sequence-conserved IncRNAs in panicles and seedlings, respectively. The internal links in gray connect IncRNAs that are sequence-conserved between O.
rufipogon, japonica and indica. (b) Two examples showed multiple lncRNAs in wild rice are sequence-conserved with rare lncRNAs in cultivated rice. Purple lines indicate 41 lncRNAs in *O. rufipogon* are homologous with 1 and 3 lncRNAs in *japonica* and *indica*, respectively. Green lines indicate 21 lncRNAs in *O. rufipogon* are homologous with 1 and 2 lncRNAs in *japonica* and *indica*, respectively. (c) Circos representation of positionally conserved lncRNAs between *O. rufipogon*, *japonica* and *indica*. Links depicted conserved synteny of lncRNAs between at least two genomes, with blue lines highlighting the eight positionally conserved lncRNA-matches between *O. rufipogon*, *japonica*, and *indica*.

2.4. LncRNAs Are Potential Targets for the Selection of Yield-Related Traits during Domestication

LncRNAs have been identified to be important in plant reproductive development and stress responses [19,29]. To investigate whether the conserved rice lncRNAs during domestication would contribute to agronomic traits, we collected mutants of several conserved lncRNAs to detect their phenotypes. We retrieved the worldwide main rice mutant databases, including Rice Mutant Database [30] and POSTECH [31], and identified three rice mutant lines with T-DNA insertion mutations in three conserved lncRNAs MSTRG.19480, MSTRG.24689 and Os10t0479100 (Figure 5a,e,h). After the screening of homozygous lines of these mutants, we investigated the agronomic traits such as plant architecture, grain morphology, and resistance to *Xanthomonas oryzae* pv. *Oryzae* (*Xoo*) and *Xanthomonas oryzae* pv. *oryzicola* (*Xoc*) pathogens. The results showed that the yield-related traits like panicle architecture, seed-setting rate, grain weight, and size, as well as the performance of these mutants in bacterial resistance, were significantly affected by the expression of conserved lncRNAs in domestication (Figure 5, Table 2 and Supplementary Figure S3).

![Figure 5](image-url) Figure 5. Phenotype analysis of the T-DNA insertion mutants of three conserved lncRNAs in rice domestication. (a–d) The diagram of T-DNA insertion site in MSTRG.19480 (a) and grain size analysis for this mutant and its wild-type Dongjin, the scale bar for grain length (b), grain width (c) or grain thickness (d) is 2 cm. (e–g) The diagram of T-DNA insertion site in MSTRG.24689. (e) and phenotype analysis for this mutant and its wild-type Dongjin. The scale bar is 5 cm for panicle (f) and 2 cm for grain length (g). (h,i) The diagram of T-DNA insertion site in Os10t0479100 (h) and phenotype analysis for this mutant and its wild-type Hwayoung. The scale bar for panicle (i) is 5 cm.
Table 2. Agronomic traits of mutants of three conserved lncRNAs during domestication.

| lncRNA ID | WT-Dongjin | MSTRG.19480 | MSTRG.24689 | WT-Hwayoung | Os100479100 |
|-----------|------------|-------------|-------------|-------------|-------------|
| Mutant ID (wild-type) | | | | | |
| Dongjin (DJ) | 1B-19117 (DJ) | 2D-40249 (DJ) | Hwayoung (HY) | 1C-07927 (HY) | |
| Plant height (cm) | 64.38 ± 3.56 | 51.38 ± 3.87 (**)↓ | 59.62 ± 4.45 (**)↓ | 64.50 ± 4.54 (**)↓ | 56.92 ± 3.04 (**)↓ |
| Panicle length (cm) | 16.80 ± 0.56 | 16.62 ± 2.04 (n.s.) | 15.76 ± 1.14 (**)↓ | 16.35 ± 1.44 (n.s.) | 15.73 ± 1.32 (n.s.) |
| Number of primary branches | 6.57 ± 0.82 | 6.06 ± 1.57 (n.s.) | 6.67 ± 0.85 (*↑) | 5.82 ± 0.57 (n.s.) | 6.57 ± 0.82 (n.s.) |
| Seed-setting rate (%) | 90.52 ± 5.05 | 90.61 ± 5.01 (n.s.) | 67.53 ± 5.08 (**)↓ | 95.00 ± 4.57 (n.s.) | 11.01 ± 3.93 (**)↓ |
| 1000-grain weight (g) | 25.56 ± 0.56 | 20.66 ± 2.93 (**)↓ | 26.40 ± 1.34 (**)↑ | 24.75 ± 1.21 (n.s.) | 24.80 ± 0.64 (**)↓ |
| Grain length (mm) | 7.64 ± 0.33 | 6.93 ± 0.27 (**)↓ | 8.28 ± 0.26 (**)↑ | 7.94 ± 0.29 (n.s.) | 8.02 ± 0.25 (n.s.) |
| Grain width (mm) | 3.49 ± 0.14 | 2.72 ± 0.17 (**)↓ | 3.43 ± 0.14 (n.s.) | 3.56 ± 0.12 (n.s.) | 3.61 ± 0.16 (n.s.) |
| Grain thickness (mm) | 2.33 ± 0.09 | 2.08 ± 0.19 (**)↓ | 2.31 ± 0.06 (n.s.) | 2.18 ± 0.08 (n.s.) | 2.14 ± 0.08 (n.s.) |
| Lesion length of Xoo infection (cm) | 6.56 ± 1.99 | 4.73 ± 1.68 (**)↓ | 5.71 ± 2.97 (n.s.) | 5.31 ± 2.29 (n.s.) | 9.10 ± 2.99 (**)↑ |
| Lesion length of Xoc infection (cm) | 2.11 ± 0.76 | 2.30 ± 0.22 (n.s.) | 0.47 ± 0.18 (n.s.) | 1.15 ± 0.76 (n.s.) | 2.19 ± 0.83 (**)↑ |

Asterisks indicate statistically significant differences compared with wild-type by Student’s t-test (*p < 0.05, **p < 0.01), n.s., not significant. Arrows indicate up- or down-regulation when compared with the wild-type plant Dongjin or Hwayoung. The sample numbers are n > 10 for plant and panicle architectures, n > 30 for grain traits, and n > 8 for Xanthomonas oryzae pv. Oryzae (Xoo) and Xanthomonas oryzae pv. oryzicola (Xoc) infection.

MSTRG.19480 locates at 3,117,854 bp to 3,119,120 bp of Chromosome 8 and transcribes a single-exon lncRNA of 1267 nt in length. Its homologous MSTRG.27043 in O. rufipogon also transcribes a single-exon lncRNAs of 1187 in length, which could be perfectly matched (percent identity = 100%) to MSTRG.19480. The only difference between these two lncRNAs is that MSTRG.19480 has 5 and 75 additional nucleotides in its 5′ and 3′ ends, respectively (Supplementary Figure S4). To further confirm that conserved lncRNAs are associated with domestication-related traits, we chose MSTRG.19480 for further investigation. We generated transgenic plants of MSTRG.19480 using the RNA interfering technology. The suppressed expression of MSTRG.19480 were verified in RNAi-1 and RNAi-2, two of the 20 transgenic lines (Figure 6a). To investigate the performance of these RNAi lines in domestication-related traits, we cultivated these plants together with the wild-type Nippobare. When compared with the wild-type plants, knockdown of MSTRG.19480 resulted in a decrease in plant height, grain length, grain width, and grain thickness (Figure 6b–j), showing similar phenotypes with its T-DNA insertion mutant (Figure 5b–d). The above observations suggested that the direct target selection on lncRNAs during domestication may contribute to the manipulation of agronomic traits in rice plants.
3. Discussion

Crop domestication is generally known as a model system of plant evolution. During this process, human selection drives crop evolution and gives rise to a number of desirable agronomic traits [32–35]. In the *Oryza* genus, the combination of pan-genome analyses and functional studies has facilitated the identification of many protein-coding genes that are responsible for marked morphological changes, including grain size, flowering, panicle development, plant architecture, and shattering. Recent progress has also revealed that noncoding RNAs widely exist and are essential in regulating plant growth and development [18]. The genome-wide scanning of noncoding RNAs, such as microRNAs and phasedRNAAs has been identified not only in cultivated rice but also in wild rice [15,36–39]. Much of the recent progress indicates that IncRNAs play important roles in plant development, however, limited data are available for their regulation in crop domestication. In this present study, we focused on the phenotypic variations of two cultivated rice varieties, *O. sativa* ssp. *japonica*, and *O. sativa* ssp. *indica*, and their wild progenitor *O. rufipogon*. Morphological and statistical analyses suggested that yield-related traits, such as seed-setting rate, grain size, and grain weight, are significantly increased in cultivated rice than in wild rice. To answer whether IncRNAs are also associated with domestication, we then collected samples of these three *Oryza* species and performed a genome-wide analysis to identify IncRNAs. Comparative analysis and functional study demonstrated that many conserved IncRNAs are associated with agronomic traits in rice. Our data further complemented the context of rice IncRNAs and suggested a potential role of IncRNAs for rice breeding.
Although both *japonica* rice and *indica* rice were developed from wild rice, they experienced separate domestication process from different *O. rufipogon* populations [9]. *Japonica* rice was first domesticated in southern China from one wild rice ecotype, while *indica* rice was later derived from cross-hybridized ancient *japonica* cultivars and other ecotypes of wild rice [9]. A recent study reported 3363 lncRNAs expressed in the panicle of *O. rufipogon* and *japonica* rice [21]. Nevertheless, the characteristics of lncRNAs among *indica* rice, *japonica* rice, and wild rice still need to be elucidated. We subsequently found in this study that the percentage of short and single-exon lncRNAs in wild rice was higher than in cultivated rice, showing a tendency distinct from that of mRNA. We also evidenced higher expression of lncRNAs in panicles all over the three datasets. As the changes of lncRNA length, exon numbers and expression patterns were accompanied by the rice domestication process, we hypothesized that lncRNAs might also be selected during domestication and might have crucial biological roles in regulating rice traits. Based on our following analysis of several lncRNA mutants, we have finally revealed the regulatory role of lncRNAs for domestication traits, suggesting promising prospects for the application of lncRNAs for crop improvement.

We identified 628 lncRNAs in wild rice that are homologous to 272 lncRNAs from *japonica* rice and 246 lncRNAs from *indica* rice. Further analysis showed multiple lncRNAs in wild rice are homologous to a few lncRNAs in cultivated rice, and sequence alignment of two lncRNA examples suggested they are highly identical to two transposable elements. There have been a number of longitudinal studies involving the expression of TE-associated lncRNAs during the domestication of plants. Some TE-associated lncRNAs are proved to be related to stress responses [40–42]. For example, the Arabidopsis expressed TE-lncRNA11195 is activated after abiotic stresses or ABA treatment. Genetic evidence revealed that disruption in this lncRNA could increase plant resistance to abscisic acid in root elongation and shoot fresh weight [41]. Our findings of decreasing the number of TE-associated lncRNAs during domestication raised the possibility that unexpected expression of TE-associated lncRNAs would be harmful for plants to conquer biotic or abiotic stress.

Breeding of domesticated traits such as higher productivity has led to reduced genetic diversity and enriched domestication-related genes among different rice accessions [42,43]. The proper expression of conserved lncRNAs is essential for rice. We collected the T-DNA insertion mutants of three sequence-conserved lncRNAs during domestication and found abnormal performance in yield-related traits such as panicle architecture, seed-setting rate, grain weight, and grain size, illustrating that lncRNAs are potential targets for the selection of yield-related traits during domestication. We further construct the RNAi lines of one conserved lncRNA, MSTRG.19480. Phenotypic analysis also confirmed its role in regulating seed size. These results indicated that lncRNAs might also be selected for maintaining or improving the agronomic traits in grass. Although we demonstrated the involvement of MSTRG.19480 in regulating yield-related traits, additional studies are needed to reveal the exact mechanism by which MSTRG.19480 modulates these traits.

4. Materials and Methods

4.1. Plant Materials and Growth Conditions

The common wild rice (*Oryza rufipogon*, collected from Hainan province), Nipponbare (*O. sativa* ssp. *japonica*) and 93-11 (*O. sativa* ssp. *indica*) were used in this study for sampling and RNA sequencing. Their Seeds were sterilized and then grown on MS medium in the growth chamber at 28 °C with a photoperiod of 10 h light and 14 h dark. The 3-week-old seedlings were then transplanted to a paddy field, and plants were maintained under routine management practices of water and soil nutrition during the rice-growing season of Guangzhou, China. The natural temperature of the growing season in Guangzhou ranged from 23.8–35.2 °C, and the day length ranged from 12.0 to 13.5 h. A total of thirty 2-week-old seedlings and thirty pre-emergence panicles for each *Oryza* species were
collected for the following analysis. The rice T-DNA insertion mutants of conserved lncRNAs were collected from POSTECH [31]. The mutant lines were sown together with their wild-type Dongjin or Hwayoung.

4.2. RNA Extraction, Library Construction and Sequencing

Total RNA was isolated with the Plant Total RNA Extraction Kit (Magen, Guangzhou, China) from each sample according to the manufacturer’s instructions, followed by digestion with DNase I (Takara, Dalian, Beijing, China). A total amount of 3 μg RNA per sample was used as input material for the RNA sample preparations. The ribosomal RNA was then removed by Epicentre Ribo-zero™ rRNA Removal Kit (Epicentre, Madison, WI, USA), and rRNA free residue was cleaned up by ethanol precipitation. Next, sequencing libraries were constructed using the rRNA-depleted RNA by NEBNext® Directional RNA Library Prep Kit for Illumina® (NEB, Ipswich, MA, USA) following the manufacturer’s recommendations. At last, products were purified (AMPure XP system, Beckman, Brea, CA, USA), and library quality was assessed on the Agilent Bioanalyzer 2100 system. The sequencing was performed on a HiseqTM 2500 machine (Illumina Inc., San Diego, CA, USA) and 125 bp paired-end reads were generated.

4.3. LncRNA Identification

Raw reads that contained adapters and low-quality bases with more than 10% of unknown nucleotides or with 50% of low quality (Q-value ≤ 20) bases were further removed by fastp [31] (version 0.18.0). The RNA mapped reads were removed by using Bowtie2 [31] (version 2.2.8). The resulting clean data were then aligned with the reference genomes using HISAT2 [31] (version 2.1.0) and transcripts were also reconstructed using Stringtie [31] (version 1.3.4). The reference genomes and annotation data were retrieved from the Rice Annotation Project for japonica rice (https://rapdb.dna.affrc.go.jp/download/irgsp1.html, accessed on 8 August 2020) and EnsemblPlants for indica rice (ASM465v1, https://plants.ensembl.org/Oryza_indica/Info/Index, accessed on 8 August, 2020) and wild rice (OR_W1943, https://plants.ensembl.org/Oryza_rufipogon/Info/Index, accessed on 8 August 2020). Transcripts that are longer than 200 nt, FPKM (fragment per kilobase of transcript per million mapped reads) ≥0.1 and CPC (coding potential calculator, version 0.9-r2, http://cpc.cbi.pku.edu.cn, accessed on 30 August 2020) score < 0 [31], CNCI (coding-noncoding-index, version 2) score < 0 [31] are considered as novel lncRNAs identified in this study. All the identified lncRNAs are listed in Supplementary Tables S1–S3.

4.4. Expression Analysis

The FPKM value were calculated to quantify the expression of transcripts by using RSEM software [44]. The violin plot and heatmap that showed expression levels of lncRNAs in wild and cultivated rice are generated according to the log10(FPKM+1) value of lncRNAs. The expression levels of twelve lncRNAs were also confirmed by quantitative RT-PCR, performed by using the SYBR Premix Ex TaqTM Kit (Takara, Dalian) following the manufacturer’s instructions. The rice ACTIN2 gene levels were used for normalization. LncRNAs relative expression levels were analyzed on QuantStudio™ 6 Flex Real-Time PCR System (Applied Biosystems, Foster City, CA, USA).

4.5. Conservation Analyses

The conserved regions in wild-japonica, wild-indica, and japonica-indica rice pairs were identified by BLAST with sequence alignment. The homologous lncRNA sequence number >100 nt and percentage identity >90% in wild-japonica, wild-indica, and japonica–indica were screened, and common homologous sequences appeared in all the three groups were selected as sequence-conserved lncRNAs. The positional conservation of lncRNAs was
analyzed using a method described previously [22]. The nearest upstream and down-
stream protein-coding genes are used to perform pairwise collinearity analyses based on
the genomes of wild and cultivated rice. If 4 out of 5 genes are close in their genomic loci, the
lncRNA in the region is considered to be positionally conserved lncRNAs.

4.6. Vector Construction and Rice Transformation
To generate the RNAi plants for suppressing the expression of MSTRG.19480, a 455 bp DNA fragments of MSTRG.19480 from Nipponbare was amplified by using the primer
pairs F: 5′-GTTGTACCTTGGAAATTCT-3′ and R: 5′-ACTATAATTCATTATGTTGT-3′. The PCR product was subcloned into pRNAi-35S plasmid in both the sense and antisense orientations to generate a stem-loop structure. The resulting plasmid was then trans-
formed into Nipponbare by using Agrobacterium tumefaciens-mediated rice transforma-
tion. Transgenic lines were screened by 50 mg/L hygromycin B (Biofroxx, Einhausen, Germany) in MS medium.

4.7. Plant Inoculations
To detect the resistance of rice to bacterial blight, flag leaves of the mutant lines were
inoculated with the Xoo by the leaf-clipping method as described [19]. The leaf tip is cut
with scissors previously dipped in PXO99A bacterial suspension with an OD600 of 0.2.
The blight lesion lengths were measured 14 days after infiltration. As to the resistance of
these mutants to bacterial leaf streak disease, the penetration method with a syringe was
used to inoculate flag leaves with Xoc strain. Lesions were photographed and measured
14 days after inoculation.

4.8. Phenotypic Analyses
The statistically significant differences analyses in this study were performed by Stu-
dent’s t-test. For the comparison of grain morphology of wild and cultivated rice, the
seeds were hulled before the measurement to eliminating the influence of awn length. For
the comparison of grain morphology of lncRNA mutants and their wild-type seeds, the
grains with their hull were used. All the experiments were designed with replicates to
measure phenotypic variables. The number of observations (n values) used to calculate
the mean value are marked in the figure legends and table legend as indicated.

5. Conclusions
In this study, we found a number of lncRNAs expressed in wild rice were revealed
to be shorter in length and have fewer exon numbers when compared with lncRNAs from
cultivated rice. Comparative analysis and the functional study demonstrated that many
conserved lncRNAs are associated with yield-related traits in rice. Collectively, our results
present the lncRNA characteristics and conservation during rice domestication and
demonstrate the potential to improve plant agronomic traits by manipulating the expres-
sion of lncRNAs.

Supplementary Materials: The following are available online at www.mdpi.com/1422-
0067/22/9/4742/s1, Figure S1: Validation of lncRNA expression profiles in RNA-seq by quantitative
real-time PCR, Figure S2: Comparison of resistance to Xoo (a) and Xoc (b) between wild type and T-
DNA insertion mutants of three conserved lncRNAs in rice domestication, Figure S3: Comparison
of resistance to Xoo (a) and Xoc (b) between wild type and T-DNA insertion mutants of three con-
served lncRNAs in rice domestication, Figure S4: Sequence alignment of MSTRG.19480 in japonica
rice and MSTRG.27043 in O. rufipogon, Table S1. Genomic loci and expression pattern of lncRNAs
identified from Oryza rufipogon, Table S2. Genomic loci and expression pattern of lncRNAs identi-
fied from Oryza sativa spp. Japonica, Table S3. Genomic loci and expression pattern of lncRNAs identi-
fied from Oryza sativa spp. Indica, Table S4. Sequence-conserved lncRNAs between wild and culti-
vated rice, Table S5. Positionally conserved lncRNAs between wild and cultivated rice.
**Author Contributions:** H.H. and Y.-F.Z. carried out the genome analysis and functional study and drafted the manuscript. Y.-W.Y., M.-Q.L., Y.-Z.F., and Z.Z. carried out mutant screening and functional experiments. Y.-C.Z. carried out the genome analysis; Y.-Q.C., J.-P.L., and Y.Y. conceived of the study, and participated in its design and coordination, and helped to draft the manuscript. All authors have read and agreed to the published version of the manuscript.

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