Global Quantitative Mapping of Enhancers in Rice Genome by STARR-seq

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Abstract

Identification of enhancers has been a challenge in plants. STARR-seq measures enhancer activity of millions fragments in parallel. Here we present a global map of rice enhancers quantitatively determined using STARR-seq. Most enhancers are mapped within genes, especially at the 5' untranslated regions (5' UTR) and the coding sequences. Silent and low expressed genes in genomic regions enriched with transposable elements (TEs) are frequently found containing proximal enhancers. Analysis of enhancer epigenetic features at their endogenous loci revealed that most enhancers do not co-localize with DNase I hypersensitive sites (DHSs) and are lack of H3K4me1. Clustering enhancers by their epigenetic modifications revealed that about 40% of identified enhancers carry one or more epigenetic marks. Repressive H3K27me3 is frequently enriched with positive marks of H3K4m3 and/or H3K27ac, which together may bookmark poised enhancers. Intergenic enhancers were predicted based on the location of DHS relative to genes, which overlap poorly with functionally identified enhancers. In summary, enhancers were quantitatively identified by functional analysis in a model plant genome, which provides a valuable resource for further mechanistic studies in different biological contexts.

KEYWORDS: Rice; Enhancer; STARR-seq; Quantification; Functional analysis

Introduction

Gene expression is tightly regulated, which is critical for plant development and responses to changes in environment and hormones [1]. Promoters at transcription start site (TSS) are frequently considered sufficient for the initiation and elongation of transcription, but the level of promoter-driven expression is generally low [1]. High level of gene expression requires the participation of enhancers to increase the efficiency of transcriptional initiation and elongation to produce more mRNAs, though the exact mechanisms are still poorly understood.

Enhancers are generally believed to regulate their target genes through the recruitment of transcription factors (TFs) and function in a distance- and orientation-independent manner [2]. In mammalian genomes, one gene can be regulated simultaneously by multiple enhancers or by different enhancers in different tissues or at different developmental stages. Moreover, one enhancer can also regulate multiple genes [2-5]. The lack of specific location relationship with target genes makes the identification of enhancers more difficult than promoters.

The advancements in molecular biology and computational techniques have enabled the characterization of enhancers genome-widely based on epigenetic marks [6-15] or direct measurement of transcription enhancing activity of candidate sequences [16-22]. Intergenic enhancers have been predicted in *Arabidopsis* according to the location of DNase I hypersensitive site relative to proximal genes [23]. However, the arbitrary exclusion of DNase I sites within 1.5kb upstream of TSS and gene body may exclude substantial amount of potential enhancers. So far, only a handful of enhancers have been identified in plants [24-28], let alone a genome-wide annotation of enhancers based on functional analysis.

Measuring enhancer activity of millions fragments had been successfully

achieved using STARR-seq in the *Drosophila melanogaster* and mammalian genomes [16, 18-20, 22]. We used STARR-seq to map potential enhancers globally in a rice genome. The accuracy of large scale quantification analysis of candidate sequences was demonstrated in a plant system. Furthermore, we revealed some epigenetic characteristics which may be unique for identified enhancers in rice genome. Finally, our results provided a regulatory element resource for further functional and mechanistic studies in different biological contexts.

Results

Global quantitative enhancer discovery using STARR-seq

To comprehensively identify sequences with enhancer activity, we constructed a reporter library from randomly fragmented genomic DNA of rice cultivar Nipponbare (*Oryza sativa L. ssp japonica*). The plasmids DNA of reporter library were transfected into protoplasts isolated from the stem of rice seedlings grown for 2 weeks in replicates and plasmids and mRNA were isolated 16 hours later. Sequencing libraries of plasmid and mRNA were generated and sequenced on an Illumina platform (**Figure 1A**). For two transfections, 15.5 million and 30.6 million (sTable 1) independent fragments were recovered with a median length of ~670 base pairs from input plasmid libraries (sFigure 1A) by Illumina paired-end sequencing. It covered ~90% of both repetitive and non-repetitive genome sequences with at least one unique fragment (sFigure 1C and 1E). For the cDNA libraries generated from isolated mRNAs, 6.1 million and 13.7 million of independent fragments were produced (sTable 1). The libraries were quality checked and the enrichment of cDNA over input plasmid was determined for each 600 bin and potential enhancers were identified (sFigure 2-6) and an example was shown in **Figure 1B**.

This method yielded 15,208 and 12,210 regions (**Figure 2A**) that were significantly enriched [$p < 0.001$] from two replicates, respectively. Pearson correlation

coefficient for the two replicates is 0.604 (sFigure 4), which indicates that STARR-seq in plant system is reproducible. Only enriched peaks identified in both biological replicates, totally 9,642 sites (sList 1), were kept for further analysis (**Figure 2A**). To validate the identified peaks, we tested 22 sites (sList 2) chosen across a wide range of enrichments by cloning them into luciferase reporter vector and quantified the reporter gene expression by reverse transcription and real-time PCR (RT-PCR) and normalized against the expression of co-transfected Renilla reporter gene. STARR-seq enrichment and RT-PCR quantification were strongly related from the entire activity range for enhancers from weak to strong (Pearson correlation coefficient, $r = 0.79$) (**Figure 1C**). The activity of the low, medium and strong enhancers also showed significant difference ($p < 0.05$ and $p < 0.001$ for comparisons between different groups, Wilcoxon test) (**Figure 1C**).

129

130 **Enhancers are enriched within gene body**

STARR-seq identified enhancers in *Drosophila* genome are mostly located within genes and at promoter regions, especially in the introns (55.6%). Only 22.6% are in intergenic regions. *Drosophila* enhancers are significantly underrepresented in repetitive sequences [16]. Different from *Drosophila*, a little more than 50% of enhancers are mapped to repetitive sequences related to TEs (**Figure 2B**) and nearly all of these enhancers (4,831/5,020) contain repetitive elements including SINE, LINE, LTR, DNA transposones, satellite DNA and simple repeats (sTable 2). Different from animal genome, repetitive sequences in rice genome are gene-rich. The enrichment of enhancers in TE-related sequences in rice may be consistent with theories that TEs may regulate gene expression or even give rise to new genes during evolution [29, 30]. Identified enhancers are overrepresented in the 5' untranslated regions (5'UTR) and coding sequences, while obviously underrepresented in introns (**Figure 2C**), which are strikingly different from the distribution pattern of enhancers in the *Drosophila* genome [16]. We divided the rice genome in two type, TE (repetitive sequences enriched with TEs) and non-TE (non-repetitive sequences) regions and analyzed the enhancer distribution relative to genes in these two types of

147 sequences. Overall, the enhancer distribution patterns are quite similar in both TE and
148 non-TE regions independent of the difference in gene activity in these two types of
149 sequences (**Figure 2D**). Enhancers locate mostly within genes at the 5' prime and
150 gradually drop to background level toward the 3' end of gene (**Figure 2D**).

151

152 **Proximal enhancers are absent from most genes in the rice genome**

153 Majority enhancers are mapped within or close to genes, which suggests that proximal
154 regulation by enhancer may be a prevalent choice in the rice genome. Compared to
155 the total number of annotated and predicted genes (~56,000) [31], the number of
156 enhancers is relatively low (9,642), less than 0.2 enhancer per gene averagely,
157 suggesting most genes may be not directly regulated by enhancers in proximity
158 (within TSS-5kb to TTS+5kb). Further analysis shows that 28.6% of genes (15,997)
159 (**Figure 3A**) with at least one enhancer in proximity, suggesting one enhancer may be
160 in close distance to multiple genes.

161 We further separated genes into four groups according to their expression level
162 (silent, low, medium and high) and calculated the percentage of genes with enhancer
163 in proximity for each group. The percentage of genes with enhancer in proximity
164 changes little for genes of different expression level in non-TE regions, and is slightly
165 increased for genes in silence or expressed at low level in TE regions (**Figure 3B**).
166 These results suggest STARR-seq enhancers at their endogenous loci are not enriched
167 at actively transcribed genes, consistent with the episomal nature of the reporter
168 plasmids.

169

170 **Genes in repetitive sequences are enriched with enhancers**

171 Repetitive sequences related to TEs are generally transcriptionally inactive, which
172 make up 42.8% of the rice genome (**Figure 2B**) and harbors 15,839 (28.3%) of total
173 genes (**Figure 3A**, left panel). However, 52.1% of identified enhancers are located in
174 TE regions (**Figure 2B**). These together suggest that genes in repetitive sequences are
175 more enriched with enhancers. Actually, 39.6% of genes (6,277) located in TE regions
176 contain at least one enhancer in proximity (**Figure 3A**, left panel). 96.7% of genes

(15,318) in TE regions are of low expression level or silent and 40.1% of them (6,141) contain at least one enhancer in proximity. For genes of higher transcription levels or genes in non-TE regions, the percentage of genes with proximal enhancer is lower than the average for total genes (**Figure 3B**, right panel). Gene ontology (GO) analysis showed that genes in TE regions with enhancers in proximity occupy the top 10 most significantly enriched categories, which are related to DNA replication, integration, nucleotide binding, etc. (sFigure 7).

185 **Most identified enhancers are not DNase I hypersensitive sites (DHSs)**

DNase I hypersensitivity has been generally believed to be associated with sequences of activities, one possibility is transcription enhancing. 8.8% of all identified enhancers overlap with DHSs (**Figure 4A**). Genes with proximal enhancer overlapping with DHS in both TE and non-TE regions show higher expression level (**Figure 4B**). Similarly, only about 13.6% of enhancers identified in the human genome co-localize with DHSs (sTable 3) [20]. Differently, 48.5% of enhancers identified in *Drosophila* co-localize with DHSs (sTable 3) [16]. The lack of DHS for the majority of enhancers proximal to actively transcribed genes may suggest that hypersensitivity to DNase I digestion may not be a universal mark for all functional enhancers in rice genome.

197 **H3K4me1 is not a ubiquitous enhancer mark in rice genome**

Histone modification mark H3K4me1 has been frequently used for enhancer prediction, which is enriched at enhancers identified by STARR-seq in both *Drosophila* and human genomes [16, 20]. However, for enhancers identified in rice, H3K4me1 is even less enriched than DHS, only ~330 sites show strong signal of H3K4me1 (**Figure 5A**). H3K4me1 is nearly completely absent from predicted enhancers based on DHS locations in the genome (**Figure 6A** and sFigure 8).

205 **H3K4me3 is enriched at many identified enhancers**

H3K4me3 is generally associated with actively transcribed genes. A significant

portion of enhancers locate inside active genes at 5' UTR and in coding sequences (Figure 2D and Figure 3). Consistently, H3K4me3 is found obviously enriched for enhancers identified, more obvious than any other histone mark or DHS (Figure 4C & 4D, Figure 5A and sFigure 8).

H3K27ac is an enriched enhancer mark

Another histone modification mark frequently used for the prediction of active enhancer is H3K27ac, which has also been shown significantly enriched at enhancers identified by STARR-seq in both *Drosophila* and human genomes [16, 20]. Different from H3K4me1, H3K27ac is also enriched for identified enhancers in rice genome, the signal is especially strong for enhancers associated with DHS or located within non-TEs (red lines in Figure 4C & 4D, Figure 5A and sFigure 8). Even enhancers not associated with DHS show slightly enrichment of H3K27ac (blue line in Figure 4C).

H3K27me3 is enriched at a significant number of identified enhancers

H3K27me3 is mostly associated with repressed genes. Surprisingly, we found that a significant number of identified enhancers are enriched with H3K27me3 (Figure 4C & 4D), which is generally absent from enhancers identified in *Drosophila* and human genomes [16, 20]. Most of these enhancers are enriched strongly with H3K4me3 and H3K27ac as well (Figure 5A and sFigure 8). That whether these enhancers are poised or actively regulating genes is difficult to determine. Different from H3K27me3, H3K9me3 is nearly completely absent from identified enhancers (Figure 5A and sFigure 8).

Epigenetic clustering of identified enhancers

We further grouped all enhancers into eight clusters based on the signal strength of multiple epigenetic marks including DHS, H3K4me1, H3K4me3, H3K27ac, H3K9me3 and H3K27me3 (Figure 5A and sFigure 8). Enhancers of cluster 1-7 (C1, sC2, C3-5, sC6 and C7; total 3,650 enhancers, 37.9% of total enhancers identified;

sC2 and sC6 are unique for STARR-seq identified enhancers) are enriched with at least one epigenetic mark (**Figure 5A** and sFigure 8). However, 62.1% of enhancers (5,992) are devoid of any analyzed epigenetic mark (C8, data not shown). H3K4me3 is strongly enriched for 4 clusters of enhancers which at the same time are enriched with different levels of H3K27ac (**Figure 5A** and sFigure 8). Overall, H3K4me3 and H3K27ac are mostly enriched, DHS and H3K27me3 are medially enriched, H3K4me1 and H3K9me3 are least enriched for enhancers of C1-7 (**Figure 5B & 5C** and sFigure 8). About 17% and 83% of C1-7 enhancers locate inside TE and non-TE regions, respectively (**Figure 5D**). Accordingly, majority of C8 enhancers (73%) are associated with TE regions (**Figure 5D**). Genes associated with each cluster of enhancers are expressed at significantly ($p < 0.01$, Wilcoxon test) higher level in non-TE regions than in TE regions except for cluster 7 ($p = 0.916$) (**Figure 5E**). Enhancers in cluster 7 are enriched with H3K27me3 (**Figure 5A**).

Predicted enhancers based on DHS location overlap little with STARR-seq identified enhancers

Enhancers had been predicted based on chromatin accessibility in *Arabidopsis* [23]. We followed the published methods and defined a DHS as enhancer if it locates beyond 1.5kb upstream of TSS and at the same time is not in a gene body. By this method, 13,770 out of total 37,168 DHSs were predicted as enhancers (**Figure 6A**, sList 3). Only 20% of them are in TE regions and 80% of them are in non-TE regions (**Figure 6B**), consistent with the fact that the repetitive sequences are mostly in closed chromatin states with low accessibility. Due to the different distribution pattern, DHS predicted enhancers overlap with only a few STARR-seq enhancers (**Figure 6C**). Strikingly, clustering of predicted enhancers (DHS signal omitted and the C8 is different from STARR-seq enhancers C8, dC2 and cC6 are also unique) showed that even less of them are enriched with histone modification marks (**Figure 6D**). Overall, the expression of genes with proximal DHS predicted enhancer in TE and non-TE regions differ little ($p > 0.05$, Wilcoxon test) except those being associated with enhancers in clusters of dC2 and dC6 ($p < 0.01$, Wilcoxon test, **Figure 6E** and sFigure

8). Moreover, except dC6 and C8, genes with enhancers in proximity are not expressed significantly higher than the middle level of total genes (**Figure 6E**).

We further compared the distribution of STARR-seq enhancers, predicted DHS enhancers and other DHS sites (with DHS predicted enhancers excluded, non-predicted enhancer DHSs, sList 4). These three groups of elements show sharply different distribution patterns relative to the TSS of genes (**Figure 6F**). As previously shown, STARR-seq enhancers are mostly enriched within gene body favoring the 5' (**Figure 6F**, left). Most DHSs are closely positioned to TSSs. In fact, 91% of all DHSs in rice genome are mapped within TSS $\pm$ 5kb regions, and similarly, 80.9% in *Drosophila* genome (sFigure 9). DHS predicted enhancers (13,770 out of total 37,168 DHSs genome-wide, 37%) are at least 1.5kb upstream away from TSS and out of gene body (**Figure 6F**, middle). For non-predicted enhancer DHSs (23,398 out of total 37,168, 63%) (sFigure 10a), they are predominantly enriched at upstream of TSS where promoters are located (**Figure 6F**, right), and only 1,347 of them overlap with STARR-seq enhancers (sFigure 10b). Due to close positioning to TSSs, significant portion of these DHSs overlap with sequences within the 200 bp upstream of TSS, $\pm$ 50bp of TSS and the 5' UTR regions (sFigure 10c), which is dramatically different from both DHS predicted and STARR-seq enhancers, respectively. Moreover, clustering on epigenetic modifications revealed that 50.5% of these sites are enriched with at least one type of modifications and most of them are enriched with H3K4me3, an active mark for active genes (sFigure 8, right). Though the majority of DHS sites are located not far from genes, these sites are less enriched inside gene body, where most of the identified enhancers are located.

Discussion

Enhancer prediction relies heavily on chromatin epigenetic mark. Though several histone modifications (H3K4me1 for most enhancers, H3K27ac for active enhancers, and coexistence of H3K27me3 and H3K4me1 for poised enhancers) have been

297 frequently used for enhancer analysis, this method may fail to predict enhancers in
 298 chromatin devoid of histone modifications. Moreover, enhancers predicted by
 299 epigenetic marks are difficult to verify by genetic methods at large scale. To measure
 300 enhancer activity of candidate sequences independent from chromatin context,
 301 STARR-seq was invented [16] and had been successfully applied to the enhancer
 302 analysis in both the *Drosophila* and human genomes [16, 20]. However, a
 303 genome-wide functional analysis of enhancers for a plant genome has not been
 304 reported, which severely limits our understanding of the nature and functional
 305 mechanisms of plant enhancers.

306
 307 Previous work predicting enhancers by chromatin sensitivity to DNase I digestion
 308 excluded arbitrarily DHSs within 1.5kb upstream of TSS and in gene body [23]. In
 309 *Drosophila*, the majority of STARR-seq enhancers are actually located within gene
 310 body or in proximity to genes [16]. Similarly, our STARR-seq analysis of rice
 311 genome also shows that the majority of enhancers are localized within the gene body.
 312 The consistent observation of enhancer enrichment in the gene body in two
 313 evolutionarily far-separated genomes may suggest that most genes could be regulated
 314 by DNA elements built in their sequences (**Figure 7**). Furthermore, it will be
 315 interesting to see if enhancers in genes can also activate other genes separated by long
 316 distance. A capture Hi-C using identified enhancers as anchors may reveal if in plant
 317 genome genes separated far away can be co-regulated by enhancers located within the
 318 gene body.

319
 320 Our analysis also reveals several unexpected features of identified enhancers in
 321 the rice genome. First, the majority of STARR-seq enhancers do not overlap with
 322 DHSs. DNase I hypersensitivity can be associated to any open and relaxed chromatin
 323 region, including insulators and other protein binding sites. Predicting enhancers
 324 relies solely on DHS location relative to the gene may fail to exclude DHSs which
 325 may possess non-enhancer activity.

326

327 Second, though H3K4me1 has been used to predict enhancers in mammals, it is
328 actually devoid from most STARR-seq enhancers and DHSs (independent of their
329 location) in rice genome. The fact that H3K4me1 is depleted from most DHSs further
330 confirms that H3K4me1 may not be the ideal mark of enhancers in rice genome.

331

332 Third, many identified enhancers are enriched with H3K27me3. The majority of
333 these enhancers are co-enriched with active chromatin marks of H3K4me3 and
334 H3K27ac. Co-existence of H3K27me3 and H3K4me3 can be explained by the theory
335 that many enhancers are poised. Surprisingly, H3K27ac and H3K27me3 co-exist at
336 quite a few enhancer sites. These two modifications are supposed to be mutually
337 exclusive. At this time, it is difficult to rule out possibilities that histones at these
338 enhancers may be modified differently in different cells, or even differently on
339 different allele in the same cell. In either case, it requires further careful analysis to
340 reveal the underlying causes of this intriguing observation.

341

342 In summary, we presented a comprehensive enhancer activity map generated by
343 quantitative measurement using STARR-seq for an important model plant. This
344 method is especially suitable for functional analysis of small genomes. Successful
345 characterization of enhancers in different cell types will help to improve our
346 understanding of the nature of enhancers in the plant genome and sheds new lights
347 into the elusive functional mechanisms of enhancers at large.

348

349 **Materials and methods**

350 **Screening vector**

351 For STARR-seq in rice cells, we constructed a screening vector based on the plasmid
352 pPBI221 backbone and modified by introducing several sequences, which include a
353 CMV 35S mini promoter, an intron and a GFP sequence, which are arranged
354 sequentially and are underlined in the following DNA sequence.

355

356 ggcttggggtagtctcaccagtcacagaaagcatcttacggatggcatgacagtaagagaattatgcagtgtgccataa
357 ccatgagtataactgcggccaacttactctgacaacgatcggaggaccgaaggagctaaccgctttttgcacaaca
358 tgggggatcatgtaactcgccttgatcgttgggaaccggagctgaatgaagccatacacaacgacgagcgtgacaccac
359 gatgcctgtagcaatggcaacaacgttgcgcaactattaactggcgaactacttactctagcttcccggcaacaattaata
360 gactggatggaggcggataaagtgcaggaccacttctgcgctcggccctccggctggctggtttattgctgataaatct
361 ggagccggtgagcgtgggtctcgcggtatcattgcagcactggggccagatggtaagccctcccgatcgtagtattcta
362 cagcagggggagtcaggcaactatggatgaacgaaatagacagatcgctgagataggtgcctcactgattaagcattgg
363 taactgtcagaccaagtttactcatatatactttagattgatttaaaacttcatttttaattaaaaggatctaggtgaagatcctttt
364 tgataatctcatgacaaaaatccctaacgtgagtttctgctcactgagcgtcagaccccgtagaaaagatcaaaggatctt
365 cttgagatcctttttctgcgcgtaactctgctgcttgcacaaaaaaaccaccgctaccagcgggtggtttgtttgccggatc
366 aagagctaccaactcctttccgaaggtaactggcttcagcagagcgcagatacacaatactgttcttctagttagccgta
367 gttaggccaccacttcaagaactctgtagcaccgcctacatacctcgtctgctaactctgttaccagtggctgctgccagt
368 ggcgataagtcgtgtcttaccgggttgactcaagacgatagttaccggataaggcgcagcggctcgggctgaacgggg
369 ggttcgtgcacacagcccagcttgagcgaacgacctacccgaactgagatacctacagcgtgagctatgagaaagc
370 gccacgctcccgaaggagaaaggcggacaggtatccggtaagcggcagggctcggaacaggagagcgcacgagg
371 gagcttcagggggaaacgcctggtatctttatagtcctgtcgggttccgccactctgacttgagcgtcgattttgtgatgc
372 tcgtcagggggggcggagcctatggaaaaacgccagcaacgcggccttttacggttcctggccttttctggccttttctc
373 acatgttcttctgcgttatccctgattctgtgataaccgtattaccgcctttgagtgagctgataccgctcggcgagcc
374 gaacgaccgagcgcagcagtgagtgagcaggaagcgggaagagcgcccaatacgcacaaccgcctctccccgcgc
375 gttggccgattcattaatgcagctggcacgacaggttcccactggaaagcgggcagtgagcgcacgaattaatgtg
376 agttagctcactcattaggcaccccaggctttacactttatgctccggctcgtatgttgtgtgaattgtgagcggatacaa
377 ttacacaggaacagctatgacctgattacgccaagcttgggccgctcagccgcccggggcgacttcgcaagacc
378 cttctctatataaggaagttcatttcatttggagagtccggatccattaacctgcaggtaaatttctagagctctggtag
379 aaatctgagggtaaatttctagttttctccttcattttcttggtaggaccctttctcttttattttttgagcttgatct
380 ttctttaaactgatctatttttaattgattggttatggtgtaaatattacatagctttaaactgataatctgattactttat
381 ttcgtgtctctatgatgatgatgattacagaaccgacgactcgtcatgacatccactttgcctttctctccacagg
382 tgtccactcccagggtccaactgcaggtcgctgcaggcttaaccatggctagcaaaaggagaagaactcttactg
383 gagttgtcccaattcttgtgaattagatggtgatgtaaaccggccacaagttctctgtcagtgagagggtgaaggt
384 gatgcaacatacggaaaacttaccctgaagttcatctgcactactggcaaaactgcctgttccctggccaacactag
385 tcactactctgtgctatggtgttcaatgcttttaagataccgggatcatatgaaacggcatgacttttcaagagt

386 ccatgcccgaagggttatgtccaggaaaggaccatcttcttcaaagatgacggcaactacaagacacgtgctgaag
387 tcaagtttgaagggtgatacccttggttaatagaatcgagttaaaagggtattgacttcaaggaagatggcaacattctg
388 ggacacaaattggaatacaactataactcacacaatgtatacatcatggcagacaaaacaaaagaatgggaatcaa
389 agcgaacttcaagacccgccacaacattgaagatggaagcgttcaactagcagaccattatcaacaaaatactcc
390 aattggcgatggccctgtccttttaccagacaaccattacctgtccacacaatctgccctttcgaaagatcccaacg
391 aaaagagagaccacatggctccttctgagtttgaacagctgctgggattacacatggcatggatgaactgtataa
392 ctgactagcgcacgaccgggcccctggtagaaatctgaggaaccgacgactcgtctgtacaacactctttccctacacg
393 acacacgtctgaactccagctctgaattcacgcgtccaggatcgttcaaacatttggcaataaagtttcttaagattgaatcc
394 tgttgccggtcttgcgatgattatcatataatttctgttgaattacgttaagcatgtaataataacatgtaatgcatgacgttattt
395 atgagatgggtttttatgattagagtgcccgcaattatacatttaatacgcgatagaaaacaaaatatagcgcgcaaactagga
396 taaattatcgcgcgcggtgtcatctatgttactagatcgggccagtcgaccaagatctccccggaattcactggccgtcg
397 tttacaacgtcgtgactgggaaaaccctggcgttaccacacttaatcgccttgacgacacatcccccttccagctggcg
398 taatagcgaagaggcccgaccgatcgccttcccaacagttgcgcagcctgaatggcgaatggcgctgatgcgtcta
399 gagtattttctccttacgcatctgtgcggtatttcacaccgcatatggcgcactctcagtacaatctgctctgatgccgatag
400 taagccagccccgacaccgccaacaccgctgacgcgcctgacgggcttctctgctcccgcatccgcttacagac
401 aagctgtgaccgtctccgggagctgcatgtgtcagaggttttaccgctatcaccgaaacgcgcgagacgaaagggcct
402 cgtgatacgctatttttataggtaatgtcatgataataatggtttcttagacgtcaggtggcacttttggggaaatgtgcgc
403 ggaacccctatttgtttttttttaaatacattcaaatatgtatccgctcatgagacaataaccctgataaatgcttcaataat
404 tgaaaaaggaagagatgagtagtattcaacattccgtgcgccttattccctttttgcggcattttgccttctgttttgcac
405 ccagaaacgctggtgaaagtaaaagatgctgaagatcagttgggtgcacgagtggttacatgaactggatctcaaca
406 gcggtgaagatccttgagagtttgcggccgaagaacgtttccaatgatgagcacttttaaagtctgctatgtggcgcggt
407 ttatcccgatattgacggcggaagagcaactcggcgcgcacatactattctcagaatgacttggtgagtactcaccag
408 tcacagaaaagcatcttacggatggcatgacagtaagagaattatgcagtgtgccataaccatgagtataacactgcg
409 gccacttactctgacaacgatcggaggaccgaaagagctaaccgctttttgcacaacatgg

410

411 Generation of input (screening) libraries

412 We extracted genomic DNA from two weeks rice seedlings. About 125 µg of genomic
413 DNA was diluted to 50ng/µl and fragmented by sonication (Scientz II-D). DNA
414 fragments (500bp-800bp length) were size-selected, end repaired, 5'-phosphorylated

415 and 3' dA-tailed with VAHTS Mate Pair Library Prep Kit for Illumina® (VAHTS; cat.
416 no. ND104). VAHTS Adapters for Illumina was ligated to about 6µg of DNA
417 fragments using VAHTS Mate Pair Library Prep Kit for Illumina® (VAHTS; cat. no.
418 ND104) following the manufacturer's protocol. Adaptor ligated DNA was purified by
419 using GeneJET PCR Purification Kit (Thermo Scientific; cat. no. K0702) then was
420 amplified by TransStart FastPfu Fly DNA Polymerase (Transgen; cat. no. AP231)
421 with Illumina sequencing primers (fw: ACACTCTTTCCCTACACGACG & rev:
422 GACTGGAGTTCAGACGTGTGC).

423

424 We obtained linear pPBI221 by PCR amplification (95°C for 5min; then 25
425 cycles of 95°C for 20s, 57°C for 20s and 72°C for 4min; Forward primer:
426 ACACGTCTGAACTCCAGTCTGTAATTC & Reverse primer:
427 GTCGTGTAGGGAAAGAGTGTGTACA). Circular plasmids was removed by
428 digestion with DpnI (NEB; cat. no. R0176) at 37°C for 30min. PCR products were
429 recovered with E.Z.N.A.® Gel Extraction Kit (Omega; cat. no. D2500). The adaptor
430 ligated genomic DNA were recombined to linearized screening vector with
431 ClonExpress II One Step Cloning Kit (Vazyme; cat. no. C112). For each reaction,
432 100ng linearized vector and 40ng adaptor ligated DNA was used in a total volume of
433 20µl. 70 reactions were performed in total. Each of 140 tubes (100µl each) of
434 Trans1-T1 Phage Resistant Chemically Competent Cell (Transgen; cat. no. CD501)
435 was transformed with 10µl of DNA, according to the manufacturer's protocol. 140
436 transformation reactions were pooled, transferred to 4L LB_{AMP} medium, and grown to
437 OD 0.8. Plasmids were purified using E.Z.N.A.® Endo-Free Plasmid Maxi Kit
438 (Omega; cat. no. D6926) then quantified.

439

440 **Protoplast preparation**

441 The rice cultivar Nipponbare (*Oryza sativa L. ssp japonica*) seeds were moistened
442 with water in Petri dishes at room temperature to promote germination (3days) and
443 then were transferred to culture box at 37°C for about 24h, and then transplanted into
444 soil. The rice plants were grown at 28°C with a relative humidity around 60%. The

445 light/dark photoperiod is 12/12 h by using a bank of 400 W metal halide lamps. The
446 light intensity is $400 \mu\text{mol m}^{-2}\text{s}^{-1}$ at the plant height. We collected 2-week-old rice
447 seedlings for the all experiments.

448

449 Protoplasts isolation is described below. Stem tissues from 80-120 rice seedlings
450 were used. 5 seedlings were cut together into approximately 0.5 mm strips using sharp
451 razors in 0.6M D-mannitol on Petri dishes. The strips were then digested (1.5%
452 Cellulase RS, 0.6 M mannitol, 0.1% BSA, 0.75% Macerozyme R-10, 3.4mM CaCl_2 ,
453 10 mM MES, pH 5.7), and then vacuumed at -50kPa in bottle for 30 min. After that,
454 the bottle was put on oscillator for 4-5h in dark with gentle shaking (45rpm) at 28°C.
455 After digestion, protoplasts were filtered through 300 micron nylon meshes and
456 washed 3-5 times with W5 solution (125 mM CaCl_2 , 154 mM NaCl, 5 mM KCl, 2
457 mM MES, pH 5.7). Protoplasts were pelleted by centrifugation at 80g for 5min. After
458 another two wash with W5 solution (equal volume), protoplasts were then
459 resuspended in MMG solution (15 mM MgCl_2 , 0.6M mannitol, 4 mM MES, pH 5.7),
460 and placed on ice for 30 min. Protoplasts were centrifuged at 80g again for 5 min and
461 finally resuspended at a concentration of 1×10^7 cells per ml using the MMG solution,
462 determined by using a hemacytometer. All procedures were carried out at room
463 temperature.

464

465 **Plasmid library transformation**

466 For each sample, 30-40 μg of plasmid was mixed with 100 μL protoplasts ($\sim 1 \times 10^6$
467 cells) in a 2 ml tube with 110 μL of freshly prepared PEG solution [40% (W/V), 0.1 M
468 CaCl_2 , 0.6 M mannitol] added. Gently mix, then incubate in dark at 28°C for 15 min.
469 Add 800 μL of W5 solution slowly. Invert gently to mix well, then centrifugate
470 immediately at 80g for 5 min. The pelleted protoplasts were resuspended gently in
471 800 μL of W5 solution. Finally, protoplasts transfected were cultured at 28°C for 16 h
472 in dark. Reporter GFP signals were checked under fluorescent microscopy.

473

474 **Reporter cDNA libraries construction for Illumina sequencing**

475 16hrs post-transfection, cells were washed in W5 solution twice and concentrated.
476 Total RNA was extracted using TransZol™ Up Plus RNA Kit (ER501). polyA+
477 RNA fraction was isolated using VAHTS mRNA Capture Beads (N401). 5U DNase I
478 (NEB M0303S) was used to digest DNA at 37°C for 20 min.

479
480 Synthesis of first strand cDNA was carried out with TransScript One-Step gDNA
481 Removal and cDNA synthesis SuperMix (AU311 50°C for 30 min and 85°C for 5s)
482 using a specific primer (GACTGGAGTTCAGACGTGTGC) and 8ul of RNA in 20
483 reactions. All reactions were pooled. The total amount of reporter cDNA was
484 amplified for Illumina sequencing by a 2-step nested PCR strategy using the
485 TransStart® FastPfu Fly DNA Polymerase (AP231-12).

486
487 In the first PCR (98°C, 2min; then 10- 12 cycles of 98 °C for 30s, 58°C for 30s,
488 72°C for 30s; and then 72°C for 5min), 10-30ng cDNA were used for amplification
489 with 2 reporter-specific primers (Fw: 5'GACTGGAGTTCAGACGTGTGC3' & Rev:
490 5'GAGCTCTGGTAGAAATCTGAGGGTGTGC3'), one of which spans the RNA
491 splice junction of GFP intron. PCR products were purified by GeneJET PCR
492 Purification Kit (K0701) and eluted in 20-30ul Elution Buffer (EB). Purified PCR
493 product as template for the second PCR (98°C for 2min; followed by 6-10 cycles of
494 98°C for 30s, 65°C for 30s, 72°C for 30s; and then 72 °C for 5min) with TransGen®
495 Biotech FastPfu Fly DNA Polymerase (AP231) and with VAHTS™ DNA Adapters
496 for Illumina® (N302). PCR products were purified by GeneJET PCR Purification and
497 eluted in 20-30 ul EB.

498 499 **Transfected plasmid library construction for Illumina sequencing**

500 When the PolyA RNA was capture, keep the total RNA (without mRNA) supernatant,
501 add 10ul RNase A TransGen® Biotech GE 101 20mg/ml at 37°C for 30-60min, and
502 the plasmid was purified by GeneJET PCR Purification Kit and eluted in 50 ul EB.
503 Purified plasmid as template for the second PCR (98°C for 2min; followed by 8-12
504 cycles of 98°C for 30s, 65°C for 30s, 72°C for 30s; and then 72 °C for 5min) with

505 TransGen® Biotech FastPfu Fly DNA Polymerase and with VAHTS™ DNA Adapters
 506 for Illumina® (N302). PCR products were purified by GeneJET PCR Purification and
 507 eluted in 20-30 ul EB. All the libraries were sequenced on Illumine X Ten platform.

508

509 **Computational analysis:**

510 **Mapping Illumina sequences**

511 For 150mer paired-end reads generated by Illumina X Ten platform, bowtie2 [32] was
 512 used to map them to the Nipponbare reference genome (IRGSP1.0). The default
 513 parameters were used except parameter “--no-discordant -X 2000”. After mapping,
 514 we used the samtools [33] to filter the mapped reads and kept only uniquely mapped
 515 reads with parameter “view -bS -f 2 -q 5”.

516

517 **Computational processing of STARR-seq data**

518 Unique fragments were used to compute the genome coverage in non-repeat region
 519 and the enrichment of cDNA signal over plasmid input. Detailed procedures to
 520 identify enhancers were described in Arnold et al (2013) [16]. And the program used
 521 in this experiment was an R package named BasicSTARRseq. We performed
 522 bonferroni correction to correct p values. Peaks are kept with p-value smaller than
 523 0.001 and enrichment fold signal over 1.3. Only enhancers found in both replicates
 524 were kept and used for further analysis. The overlapped enhancers were merged by
 525 bedtools merge [34].

526

527 **SATRR-seq enhancers overlap with DNase I hypersensitive sites (DHSs)**

528 We consider it is an overlap if the middle point of an enhancer falls in a DHS site. By
 529 this definition, 848 enhancers were found overlapping with DHS. DHS data were
 530 generated by Zhang et al. [35]. DHS open sites were called by MACS1.4 [36] with
 531 default parameter.

532

533 **DHS predicted enhancers according to their location relative to genes**

534 Enhancers were predicted in the rice genome following the definition published

535 previously for enhancer prediction in Arabidopsis [23]. DHSs located in intergenic
536 regions (>1.5 kb upstream of TSS and downstream of transcription terminal site, TTS)
537 were arbitrarily defined as enhancers. Total 37,168 DHSs were called by MACS1.4 in
538 the whole rice genome. We used bedtools intersect to filter the DHSs in intergenic
539 regions and 13,770 DHSs were considered as predicted enhancers.

540

541 **Correlation analysis between different libraries**

542 Reads inside consecutive 500bp bins were used to calculate the correlation between
543 different libraries. Bins with value of zero were excluded from the calculation. We
544 used multiBamSummary and plotCorrelation in deeptools [37] with parameter:
545 --binSize 500 --ignoreDuplicates, and -corMethod pearson --skipZeros. Pearson
546 correlation coefficients were calculated.

547

548 **Coverage analysis**

549 Only unique fragment data were used to compute the read coverage at each position
550 for both repetitive and non-repetitive genome sequences. We used bedtools to
551 calculate the coverage ratio.

552

553 **Enhancer distribution in the genome**

554 We used MSU 7.0 to annotate the rice genome in the following categories: repeat,
555 coding sequence, core promoter (+/- 50 bp TSS around the TSS), 5' UTR, 3' UTR, 1st
556 intron, intron, 200bp upstream TSS, intergenic. Each enhancer was sorted into
557 different category if its summit falls within sequence of a specific category. The
558 distance between enhancer and proximal TSS was computed by bedtools closest
559 command.

560

561 **Analysis of GC-content effect of plasmid/cDNA libraries**

562 We calculated the GC content for each non-overlapping 100bp bins in the non-repeat
563 genome. Sequences were divided into 100 bins from 20% to 80% according to their
564 GC content. The cDNA/plasmid mean read coverage in each bin was also calculated.

565 The softwares used are makewindows, nuc, coverage of bedtools suit.

566

567 **Intersecting genomic coordinates**

568 We used bedtools suite of programs to intersect genomic coordinates for STARR-seq
569 enhancers, DHS enhancers and the closest TSS to a specific enhancer.

570

571 **STARR-seq, DHS-seq, and chromatin mark enrichment**

572 Histone modification data, including H3K4me3, H3K27me3 were retrieved from
573 GSE19602 [38], and the H3K27ac, H3K9me3 were retrieved from GSE79033 [39],
574 the H3K4me1 was retrieved from GSA under accession no. PRJCA000387 [40]. DHS
575 data were from GSE26734 [35]. All the histone modifications were mapped to the
576 reference genome IRGSP 1.0 using bowtie2 with parameter --no-discordant -X 2000
577 for paired-end data and --no-discordant for single-end data, and only the uniquely
578 mapped reads were kept with duplicates removed by picardtools. The histone mark
579 peaks were called by MACS1.4 with default parameters. Adapter was removed from
580 the DHS sequencing data by cutadapt before mapping. Duplicates were removed with
581 picardtools, and peaks were called by MACS1.4. Reads density at each position was
582 normalized for comparison. 10,000 randomly selected regions of 700bp were used as
583 control, and repeated for at least 10 times to calculate the mean value. The enrichment
584 of chromatin mark and DHS were showed in a 5kb window with the enhancer peak
585 positioned in the center. STARR-seq enhancer, DHS enhancer and DHSs except
586 predicted enhancers were divided into two groups depending on if they were located
587 in repeat regions. STARR-seq enhancers were also divided into two groups according
588 to their location relative to DHS. We used the R package EnrichedHeatmap [41] to
589 plot the histone modifications (H3K4me1, H3k27ac, H3k4me3, H3K9me3,
590 K3K27me3), and DHS enrichment for all groups with the center of analyzed elements
591 positioned at middle point and extended to upstream and downstream by 5kb,
592 respectively.

593

594 **Determine the proximity of enhancer to genes**

An enhancer is defined in proximity of a gene if it is located within the range of gene body +/- 5kb region. Genes were separated in two groups, TE and non-TE according to their location in either TE or non-TE regions. The number of TE gene is 15,839, and the number of Non-TE gene is 39,961. The tools we used is BedTools. Gene expression data are from Zhang et al. (2016) [31].

GO analysis of genes with enhancer in proximity

We used agriGO V2.0 [42] for the GO enrichment analysis.

Histone modification at enhancers

Relation between histone modifications, DHS and enhancers were analyzed. 3,650 out of total 9,642 STARR-seq enhancers overlap with at least one type of histone mark or DHS. 2,331 out of 13,770 DHS enhancers overlap with at least one kind of histone mark, while 11,819 out of 23,398 DHS peaks (with DHS enhancers excluded) overlap with at least one type of histone mark.

Enhancer categorizations based on epigenetic marks

Enhancers were classified into 8 clusters based on histone marks, DHS and relative enrichment of these marks. K-Means in Cluster3.0 [43] was used. Expression level of proximal genes with enhancers of each category was analyzed.

Distribution of STARR-seq enhancers relative to the gene body

TSSs and TTSs were aligned and sequences between were equally divided into 10 bins. Another 2.4kb was extended upwards and downwards from TSS and TTS, respectively. The 2.4kb extended regions were divided into 10 equal sized bins as well. The size of 2.4kb was arbitrarily chosen for it is the median size of all genes in rice genome. 55,000 regions of 2.4kb were randomly generate by bedtools random command with parameter -n 55000 -l 2400, which were repeated for 100 times. Genes were separated in TE and non-TE groups. We counted the enhancer number in each bin and normalized against the total number of genes in each category or 55,000 for

625 random control.

626

627 **Percentage of genes with enhancers in proximity**

628 Genes were divided into four groups based on their expression level. High,
629 RPKM >10; Medium, $1 < \text{RPKM} \leq 10$; Low, $0 < \text{RPKM} \leq 1$ and Silent. Genes were
630 separated into two groups, TE and non-TE, to compare.

631

632 **Motif analysis**

633 For each group of elements, we submitted their sequences to the MEME-ChIP web
634 server¹⁹ for de novo motif finding, using default parameters. The database we used
635 was JASPAR CORE (2018) 20 plant database. Identified motifs are enclosed in
636 supplementary List 5.

637

638 **Statistical analysis**

639 We used R for all statistical analysis.

640

641 **Data Availability**

642 All data are available under the accession number GSE121231.

643

644 **Authors' contributions**

645 JS constructed the reporter library and validated identified sites. LN designed and
646 monitored the experiments. YZ participated in cell preparation, transfection and
647 sequencing library preparation. NH carried out bioinformatics analysis. WS processed
648 the raw data and participated in bioinformatics analysis. YZ helped with reporter
649 library construction. LL advised on data analysis. CH designed, monitored the
650 experiments and wrote the manuscript with input from all authors.

651

652 **Competing interests**

653 The authors have declared no competing interests.

654

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663

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803 **Figure legends**

804

805 **Figure 1 Genome-wide quantitative enhancer discovery**

806 **A.** STARR-seq work flow. CMV 35s mini promoter was used to drive basal
807 expression of reporter gene [ORF, open-reading frame; pA site, polyadenylation site].
808 **B.** STARR-seq cDNA (red) and input plasmid (grey) fragment densities in a genomic
809 locus. Black boxes denote identified enhancers in two replicates. **C.** STARR-seq and
810 Real-time PCR quantification are linearly correlated: r , Pearson correlation coefficient;
811 Error bars indicate two independent biological replicates; Inset, the same data
812 depicted in box plot; * $p < 0.05$, *** $p < 0.001$, Wilcoxon rank-sum test.

813

814 **Figure 2 Genomic distribution of STARR-seq identified enhancers**

815 **A.** Enhancers identified by two independent STARR-seq experiments. 9,642
816 enhancers were discovered in both replicates. **B** and **C.** Distribution and relative
817 enrichment of identified enhancers in the rice genome. **D.** Distribution of enhancers
818 relative to the gene body. TSS (transcription start site) and TTS (transcription
819 termination site) are aligned and gene bodies are divided into 10 equal bins. Extended
820 from TSS to upstream and from TTS to downstream are 10 bins of equal size (240bp,
821 determined by dividing the median gene size by 10), respectively.

822

823 **Figure 3 Presence of identified enhancers in proximity to genes of different** 824 **expression level**

825 **A.** Number of genes expressed at different levels with or without enhancers in
826 proximity in TE (left) or non-TE regions (right), respectively. Enhancers within the
827 range from TSS-5kb to TTS+5kb are defined as in proximity to genes. **B.** Percentage
828 of genes expressed at different levels in TE (left) or non-TE regions (right) with or
829 without enhancers in proximity. Silent, RPKM=0; Low, $0 < \text{RPKM} \leq 1$; Medium, $1 <$
830 $\text{RPKM} \leq 10$; High, $\text{RPKM} > 10$.

831

Figure 4 Epigenetic characteristics of identified enhancers at their endogenous loci

A. Numbers of identified enhancers overlap with DNase I hypersensitive sites, DHSs. **B.** Expression of genes in TE or non-TE regions with proximal enhancers overlapping with DHS or not; **** $p < 10^{-10}$, Wilcoxon rank-sum test. **C.** Epigenetic signal enrichment for enhancers overlapping with DHSs (red) or not (blue). **D.** Epigenetic signal enrichment for enhancers in non-TE (red) and TE (blue) regions, respectively.

Figure 5 Epigenetically clustered STARR-seq enhancers

A. Identified enhancers are grouped into 8 clusters based on the signal strength of 6 epigenetic marks. Numbers of enhancers in each cluster are shown at the bottom. Overall signal density was ranked: absent (close to background level) as light grey box, low as light red, medium as dark red and high as bright red. **B.** Percentage of identified enhancers in each cluster with (red) or without (light grey) indicated epigenetic mark. **C.** Identified enhancers with indicated epigenetic mark in each cluster were further divided into three sub-classes based on the average signal density, percentage of enhancers of low, medium and high signal density are shown. **D.** Percentage of cluster 1-7 and C8 in TE (light grey) or non-TE (red) regions. **E.** Expression of genes in TE (blue) or non-TE (red) regions with enhancers of different cluster in proximity. Genes in non-TE regions with enhancers of nearly all clusters (except C7) show significant ($p < 0.01$) higher expression level than in TEs.

Figure 6 Predicted enhancers based on DHSs location to genes

A. Numbers of predicted enhancers (13,770) according to DHSs location and of DHSs with predicted enhancer excluded (23,398). **B.** Distribution of predicted enhancers in the genome. **C.** Overlap of predicted enhancers and STARR-seq enhancers. **D.** Pie charts of clustered enhancers. For predicted enhancers, DHS signal was omitted for clustering. **E.** Expression of genes in TE (blue) or non-TE (red) regions with enhancers of each cluster in proximity. Genes in non-TE regions with enhancers of clusters 2, 6, 7 and 8 show significant ($p < 0.01$) higher expression than genes in TE

862 regions. **F.** Relative enrichment of identified enhancers, predicted enhancers and DHS
863 sites excluding predicted enhancers around the TSS. Red and blue lines show sites in
864 non-TE or TE regions, respectively.

865

866 **Figure 7 Proposed enhancer function model in rice genome.** Gene body is shown
867 in green. Density of identified enhancers gradually decreases within gene body from 5'
868 TSS to the 3' TTS. Genes can be self-regulated by enhancers located within.
869 Enhancers in genes may also activate other genes separated far away. Predicted
870 enhancers are mostly located away from genes and if they activate genes or not
871 requires systematic experimental validation.

872

873 **Supplementary materials**

874 Figures S1 to S10

875 Tables S1 to S3

876 Lists S1 to S5

877

878 **Supplementary Figure 1 Plasmid and cDNA libraries**

879 **A and B.** Fragment sizes of STARR-seq input (top left) and cDNA libraries (top right),
880 the median fragment sizes are indicated. **C and D.** Coverage of the rice non-repetitive
881 genome by independent fragments (cumulative), **C** for plasmid library, **D** for cDNA
882 library. **E and F.** Coverage of the rice repetitive genome by independent fragments
883 (cumulative), **E** for plasmid library, **F** for cDNA library. More than 90% of the
884 genome is covered by more than one fragment in input plasmid library.

885

886 **Supplementary Figure 2 GC content analysis for plasmid and cDNA libraries**

887 The genome sequence was binned according to GC-content (x axis). Each boxplot
888 shows the read depth of all positions within the respective windows, revealing that
889 STARR-seq input shows similar pattern as the cDNA library.

890

891 **Supplementary Figure 3** Correlation analysis of all sequenced libraries. See
892 Methods for analysis description.

893

894 **Supplementary Figure 4** Correlation analysis of enhancers from two replicates

895 For all enhancers identified by two independent biological replicates, the correlation
896 of their activity was reasonably high with a Pearson correlation coefficient $r = 0.604$.

897

898 **Supplementary Figure 5** Enhancer distribution relative to their strength

899

900 **Supplementary Figure 6** Number of unique fragments supporting enhancer
901 identification

902 Percentage of enhancers versus the number of independent genomic fragments per
903 enhancer.

904

905 **Supplementary Figure 7** GO analysis of genes mostly enriched

906 Top ten categories of enriched genes with identified enhancer in proximity. Most of
907 them are located within TE regions and are involved in the biological process of DNA
908 synthesis.

909

910 **Supplementary Figure 8** Enhancer and DHSs clustering on epigenetic signals

911 STARR-seq enhancers, DHS predicted enhancers and the remaining DHSs are
912 clustered. Note that the two groups of DHSs were clustered without the values of
913 DNase I hypersensitivity. sC2, sC6 are unique for STARR-seq enhancers. dC2, dC6
914 are unique for the two groups of DHS elements.

915

916 **Supplementary Figure 9** Distribution of DHSs relative to TSSs

917 The relative DHSs enrichment in Drosophila and rice genomes are calculated in
918 moving 500bp bins normalized against total number of bins at each position. 0 on X
919 axis corresponding to TSS.

920

921 **Supplementary Figure 10 DHSs with predicted enhancer sites excluded**

922 A. Total DHSs are divided into two groups, 13,770 sites as predicted enhancers,
923 23,398 sites within TSS-1.5kb and in gene body. B. Overlap of STARR-seq enhancers
924 and non-predicted enhancer DHSs sites. C. Relative enrichment of non-predicted
925 enhancer DHSs sites in different categories of genome sequences.

926

927 **Supplementary Table 1 Sequencing statistics of libraries**

928

929 **Supplementary Table 2 STARR-seq enhancers in TE regions overlap with**
930 **different types of repetitive sequences**

931

932 **Supplementary Table 3 Percentage of STARR-seq enhancers overlapping with**
933 **DHS**

934

935 **Supplementary List 1 STARR-seq identified enhancers**

936

937 **Supplementary List 2 Real-Time PCR validated enhancer sites**

938

939 **Supplementary List 3 Predicted enhancers according to DHS location**

940

941 **Supplementary List 4 non-predicted-enhancer DHSs**

942

943 **Supplementary List 5 Identified motifs for different groups of elements**

Figure 1, Sun et al.

Figure 2, Sun et al.

Figure 3, Sun et al.

A

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B

Figure 4, Sun et al.

Figure 5, Sun et al.

A

B

C

D

E

Figure 6, Sun et al.

Figure 7, Sun et al.