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Functional characterization of transposon-tagged abiotic stress-responsive rice genes and their molecular polymorphisms among various stress-tolerant genotypes

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Abstract *Dissociation* (*Ds*) insertional mutagenesis has been regarded as an efficient tool to generate insertion mutants for functional genomics and molecular breeding. However, little is known about the application of the tool on exploring biological functions of abiotic stress-related genes and their molecular breeding experience. In this study, a total of 833 *Ds* insertion lines have been obtained, which showed significantly higher tolerance or sensitivity to high salinity, drought or cold stress, by screening around 20,000 *Ds* lines. Analysis of *Ds* flanking sequence tags revealed that 165 rice genes were tagged by *Ds* insertion. Gene Ontology (GO) and gene set enrichment analysis showed that over-represented *Ds*-tagged genes might function in the response to exogenous stimuli. These *Ds*-tagged genes showed expression divergence among five high salinity and five drought tolerant rice lines under either high salinity or drought stress. Higher percentages of *Ds*-tagged genes were down- or up-regulated by these abiotic stresses. These *Ds*-tagged genes were also frequently reduced or suppressed by various phytohormones including abscisic acid and jasmonate. On the other hand, we have also detected single nucleotide polymorphisms (SNPs) and 1–10 base pairs of insertion and deletions (indels) of these *Ds*-tagged genes among ten high salinity/drought tolerant rice lines by comparing with the reference genome Nipponbare. Our data showed that SNPs were detected among 102 out of 165 genes and indels were identified in 39 out of 165 genes. All the data provided additional information to further explore the biological functions of these genes or to carry out molecular breeding.

Keywords Abiotic stresses · Differentially expressed genes · Insertion and deletions (Indels) · Insertional mutagenesis · *Oryza sativa* · Single nucleotide polymorphism (SNP)

Abbreviations

ABA	Abscisic acid
Ac	*Activator*
BR	Brassinosteroid
CK	Cytokinin
DEG	Differentially expressed gene
Ds	*Dissociation*
FST	Flanking sequence tag
GA	Gibberellic acid
GO	Gene ontology
GSEA	Gene set enrichment analysis
IAA	Indole-3-acetic acid
Indel	Insertion and deletion
JA	Jasmonic acid
MAS	Molecular marker-assisted selection

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PEG	Polyethylene glycol
RNA-Seq	RNA-sequencing
SNP	Single nucleotide polymorphism
TAIL-PCR	Thermal asymmetric interlaced-PCR

Introduction

Crop plants are frequently exposed to various environmental stresses such as drought, high salinity, and cold. As a result, grain yield was decreased by more than 50% in some crops (Bray et al. 2000). Therefore, it is important to develop abiotic stress-tolerant crops to minimize these losses. Currently, scientists have developed several strategies to improve crop tolerance to these abiotic stresses, which include traditional and molecular-based methods. The prerequisite to improve rice tolerance to various abiotic stresses by traditional breeding strategy is that enough breeding materials are available, which show acceptable tolerance to these abiotic stresses and can be used as parental lines during sexual hybridization. However, these breeding materials were generally from natural mutations and their mutation rate is very low at only 10^{-5}–10^{-8} in higher plants. Thus, available breeding materials are limited. On the other hand, abiotic stress tolerance was controlled by multiple genetic loci in many naturally available breeding materials. For example, the rice variety Pokkali is highly tolerant to salinity stress and was widely used for rice breeding; however, the trait was controlled by two or more genes (Elahi et al. 2004; Kavitha et al. 2012; De Leon et al. 2015). Thus, it took breeders long breeding period with low breeding efficiency for them to use these materials to develop high tolerant rice varieties. Therefore, more efficient strategies should be employed to further improve crop tolerance to these abiotic stresses.

Currently, molecular marker-assisted selection (MAS) has been widely used for crop breeding to more efficiently select higher tolerant rice lines to abiotic stresses (Das and Rao 2015). However, limited molecular markers retard the application of MAS on crop breeding. Thus, it is important to discover more molecular markers or genes that significantly contribute to abiotic tolerance. At least two strategies have been employed to discover these abiotic stress-related genes. One of them is to identify differentially expressed genes (DEGs) under stress conditions and then to verify their biological functions of these candidates in response to abiotic stresses. DEGs were usually genome-widely identified through microarray, SAGE (serial analysis of gene expression; Velculescu et al. 1995), MPSS (massively parallel signature sequencing; Brenner et al. 2000), or RNA-Seq (RNA-sequencing; Mortazavi et al. 2008) analysis. By custom-designed microarray chips, we have identified a total of 2282 up-regulated genes under high salinity stress in both salinity sensitive (Nipponbare) and tolerant (Pokkali) lines (Jiang et al. 2013). Among them, 947 and 652 genes were detected only in Pokkali and Nipponbare, respectively. On the other hand, we have identified 3253 down-regulated genes, among which 789 and 1439 genes were detected only in Pokkali and Nipponbare, respectively (Jiang et al. 2013). To verify their potential of these differentially regulated genes in improving abiotic tolerance, transgenic plants might be required by over-expressing or down-regulating these genes. Thus, a large amount of work must be carried out for the verification.

The second strategy is to artificially generate large amount of mutant population and to use them to screen for abiotic tolerant lines, from which new abiotic stress-related genes will be functionally characterized. Besides natural mutagenesis, several tools have been developed for artificial mutagenesis including physical, chemical, tissue culture, T-DNA, transposon, and gene silencing/editing-based mutagenesis (Jiang and Ramachandran 2010). Using the mutants generated from these strategies, hundreds of thousands of rice genes have been functionally characterized. For the physical, chemical, and tissue culture mutagenesis, a large amount of mutant population could be easily established. However, more works have to be carried out to genetically map and characterize the tagged genes. For gene silencing/editing-based mutagenesis, a mutant population is not easily established as the method is employed to functionally characterize single or certain genes. For both T-DNA and transposon mutagenesis, it is not difficult to establish mutant populations and targeted genes can be obtained by PCR-based methods. Thus, these two methods are suitable for establishing a mutant population, which can be used for screening to obtain abiotic responsive lines and their targeted genes.

At least 450,000 T-DNA insertion rice lines were generated, from which more than 35,000 franking sequence tags (FSTs) were obtained from coding gene regions. For transposon insertion mutagenesis, more

than 110,000 insertion rice lines were generated and around 25,000 FSTs were available but only around 4000 FSTs were located within gene regions (Jiang and Ramachandran 2010). Although a large amount of T-DNA/transposon mutant population has been established, functionally characterized genes through these mutants were limited. These might be partially due to that the limited visible phenotype or agronomical traits were available for investigation after T-DNA/transposon insertion into a single locus of the rice genome. In fact, many genes might play roles only in some stressed conditions, especially for these genes with regulated expression patterns under various abiotic or biotic stresses. On the other hand, during long evolutionary history, many genes have been evolved with redundant functions. In both cases, no visible phenotype will be observed after these genes are mutated. Thus, majority of reports were focused on functional characterization of these genes with roles in controlling visible phenotypes such as plant height (Margis-Pinheiro et al. 2005), male fertility (Zhu et al. 2004; Jiang et al. 2007), and so on. Limited data are available for screening mutants with phenotypes under stressed conditions. In this study, a total of 4496 FSTs were obtained from *Dissociation* (*Ds*) insertion lines by thermal asymmetric interlaced-PCR (TAIL-PCR; Liu and Whittier 1995) followed by sequencing. The investigation of these FSTs showed that the *Ds* element was distributed on all rice chromosomes. By comparing the insertion events in coding regions with that in non-coding regions, we found that *Ds* was preferentially inserted into exon regions of a gene. Around 20,000 *Ds* insertion lines were subjected to high salinity, drought, and cold stresses to screen for tolerant or sensitive lines to these stresses. A total of 833 lines were obtained, which showed significantly different tolerance or sensitivity to these stresses. We obtained FSTs from 202 abiotic stress-responsive lines, from which 165 genes were tagged by the *Ds* element. Further analyses showed that higher percentage of these genes were up- or down-regulated by high salinity, drought, or cold stress. They were also frequently induced or suppressed by various hormones including abscisic acid (ABA) and jasmonate (JA). These *Ds*-tagged genes also exhibited molecular polymorphisms among five high salinity and five drought tolerant rice lines. All these data provide additional information to further explore the biological functions of these genes and their potential application on rice molecular breeding.

Materials and methods

Plant materials, growth conditions, and stress treatments

Rice species *Oryza sativa* japonica variety Nipponbare, which has been genome-widely sequenced (Goff et al. 2002), was used for the generation of *Ds* insertion lines. The five high salinity tolerant lines (PI279132, PI399813, PI400694, PI439048, PI464588) and 5 drought tolerant lines (PI201839, PI224899, PI373139, PI388466, PI584612) were the gifts from Germplasm Resources Information Network, Agricultural Research Service, the US Department of Agriculture (https://www.ars-grin.gov/). Seeds from all the rice lines were germinated in water at 37 °C for 3 days and were then planted in soiled pots. The rice plants were grown in greenhouse under natural light and temperature conditions.

For both high salinity and drought stress treatments, 14-day-old plants were harvested from pots and were then washed out soil with water. The seedlings were then used for stress treatments. For high salinity treatment, plants were treated with 250 mM sodium chloride (NaCl) solution and the whole seedlings were harvested in 0- and 8-h intervals for total RNA isolation. The 30% polyethylene glycol (PEG, 6000) was applied to the seedlings, and the samples were collected in 0- and 0.5-h intervals for total RNA isolation.

Generation of *Ds* insertion lines and their screening under various abiotic stresses

The *Activator* (*Ac*)/ *Ds* tagging system was established according to the description (Kolesnik et al. 2004). The maize transposon *Ac* element was wing clipped and non-autonomous for transposition but provides a cassette for transposase. The maize *Ds* element generates no transposase but provides wing elements. The *Ac* and *Ds* lines were separately generated from *Ac* and *Ds* constructs with *GFP* (green fluorescence protein) and *Bar* (phosphinothricin acetyltransferase gene) as negative and positive selection markers by *Agrobacterium*-mediated transformation. The homozygous *Ac* and *Ds* lines were then used as parents for sexual hybridization to obtain F1 seeds. The homozygous *Ds* lines were selected in the fifth self-crossing generation by spraying herbicide Basta. These homozygous *Ds* lines were used to screen for tolerant or sensitive lines to high salinity, drought, and cold stresses.

Around 20,000 *Ds* insertion lines were subjected to these three different stresses for screening. For high salinity stress, roots of 2-week-old seedlings were submerged under 50 mM NaCl solution to obtain hypersensitive or overly sensitive lines and under 200 mM NaCl to obtain tolerant lines. PEG solution was used to mimic drought condition, and 3-week-old seedlings were submerged under 3% PEG to screen for hypersensitive or overly sensitive lines, and 10% PEG was used to screen for tolerant lines. The cold stress experiment was carried out under natural winter conditions in Zhuhai city, Guangdong province, China. The temperature ranged from 10 to 20 °C during the season. For all these stress experiments, the phenotypic survey was carried out according to the standard evaluation system for rice as described in the Rice Knowledge Bank at the International Rice Research Institute (http://www.knowledgebank.irri.org/images/docs/rice-standard-evaluation-system.pdf).

Ds flanking sequences and their tagged genes

Genomic DNA samples were extracted from leaves of both tolerant and sensitive *Ds* lines (Dellaporta et al. 1983). Genomic DNA templates were subjected to TAIL-PCR (Liu and Whittier 1995) to obtain DNA fragments flanking the *Ds* element. The DNA fragments were sequenced to obtain *Ds* flanking sequence tags. FSTs were submitted to the Rice Genome Annotation Project database (http://rice.plantbiology.msu.edu/; Kawahara et al. 2013) for BLASTN searches to identify the tagged genes.

Gene set enrichment analysis (GSEA)

Gene ontology (GO) terms of *Ds*-tagged genes were obtained from the release 7 of the rice genome annotation project database (http://rice.plantbiology.msu.edu/; Ouyang et al. 2007; Kawahara et al. 2013). To determine overrepresented genes and their gene ontology categories, we analyzed all three top GO categories including biological processes (P), molecular functions (F), and cellular components (C) (Harris et al. 2004). GSEA (Mootha et al. 2003) was used to determine over- or under-represented genes and their GO categories.

Total RNA extraction, cDNA library construction, and RNA-Seq

Total RNA samples were extracted from the 2-week-old whole seedlings using the RNeasy Plant mini kit (Qiagen) according to the manufacturer's instructions. DNase I (Invitrogen) was used to digest any contaminated DNAs in the RNA samples. After verifying the quality and purity by Nanodrop 2000 and electrophoresis on 1.0% agarose gels, the total RNA samples were used for mRNA preparation and cDNA synthesis followed by cDNA library construction. The libraries were sequenced using Illumina HiSeq 4000 by Beijing Genomics Institute (BGI).

Expression analysis of *Ds*-tagged genes under high salinity and drought stresses by RNA-Seq

After sequencing, raw reads were filtered by excluding low-quality, adaptor-polluted, and high content of unknown base (N) reads to get clean reads, which were then mapped to the reference genome Nipponbare using the program Bowtie 2 (v2.2.5, http://bowtie-bio.sourceforge.net/bowtie2/index.shtml; Langmead and Salzberg 2012). Gene expression level was estimated by the program RSEM (RNA-Seq by Expectation-Maximization, v1.2.12, http://deweylab.github.io/RSEM/; Li and Dewey 2011). Differentially expressed genes under high salinity or drought stress were identified by the program NOIseq according to the description (Tarazona et al. 2011). The parameters used for detection of differentially expressed genes were fold change ≥ 2.00 or ≤ 0.5 and statistical probability ≥ 0.9. The original results were presented in the Supplementary Table 1. The $\log_2$ (fold change) values were used for all diagram constructions.

Detection of SNPs and indels by RNA-Seq

The genome mapping results from RNA-Seq data were used for detecting SNPs and indels by the program Genome Analysis Toolkit (GATK, v3.4-0). The program was developed by McKenna et al. (2010) and was downloaded from the website https://www.broadinstitute.org/gatk. The parameters to call SNPs and indels are "-allowPotentiallyMisencodedQuals -stand_call_conf 20.0 -stand_emit_conf 20.0." The final SNPs and indels were obtained by filtering out the unreliable sites using the following parameters

(-window 35 -cluster 3 -filterName FS -filter "FS > 30.0" -filterName QD -filter "QD < 2.0"). All the SNPs and indels from five high salinity and five drought tolerant lines were identified by comparing the reference genome "Nipponbare." The original results were attached in the Supplementary Tables 2 and 3.

Microarray expression database and identification of differentially regulated genes under hormone treatments

To explore the expression profile of *Ds*-tagged genes under hormone treatments, we surveyed the transcription regulation of these genes under six different hormone treatments including ABA, brassinosteroid (BR), gibberellic acid (GA), auxin (indole-3-acetic acid, IAA), JA, and cytokinin (CK). The dataset with GEO (Gene Expression Omnibus; Barrett et al. 2013) accession GSE39429 (Sato et al. 2013) was downloaded from the NCBI GEO dataset (https://www.ncbi.nlm.nih.gov/geo/). Each expression raw data is applied to (1) 75 percentile normalization and (2) $\log_2$ transformation. The $\log_2$ value was used for all diagram construction. Genes with at least two times difference in their normalized signal value based on computing geometric mean between control and treatments were submitted for Student's *t* test. These genes with a statistical difference at $P < 0.05$ were regarded as differentially regulated genes in their expression.

Results

Generation of around 20,000 *Ds* insertion lines and their flanking sequence tag (FST) analysis

A two-element *Ac*/*Ds* gene trap system was established in rice (Kolesnik et al. 2004; Jiang et al. 2007), which was used to generate a mutagenesis population consisting of around 20,000 *Ds* insertion lines. Previously, we reported the chromosome distribution of 1072 FSTs (Kolesnik et al. 2004). Now, we have obtained 6859 FSTs, from which 4496 FSTs were unique (Fig. 1a). Among the 4496 FSTs, 3029 FSTs were mapped on rice chromosomes while the rest were from either T-DNA sequences or contamination. Among the 3029 FSTs, 1303 of them were located at gene regions and 1723 at non-gene regions. We are interested in the 1303 FSTs which were mapped on gene regions (Fig. 1a). Among these FSTs, the majority of them (1112, 85%) were located on coding regions and the rest (191, 15%) were in the intron regions (Fig. 1a).

To evaluate the characterization of *Ds* insert into rice genome, we carried out various comparisons. In the rice Nipponbare genome, the total genome size of annotated genes is around 166 Mb and the whole genome size is around 373.2 Mb. Thus, 44.5% of the genome is used for encoding genes (Fig. 1b). Among 3026 FSTs, 43.1% of them were located at the annotated gene regions (Fig. 1b). No significant difference was observed between these two percentages (light and dark blue columns in Fig. 1b). In the rice genome, the percentage of the total cDNA size of annotated genes (113.3 Mb) among the whole genome size (373.2 Mb) is 30.4% (light green column in Fig. 1b). However, our data showed that up to 36.7% of FSTs were mapped on cDNA regions (dark green column in Fig. 1b), significantly higher than 30.4% by *Z* test ($p = 0.0476$). On the contrary, up to 14.1% of the rice genome is occupied by intron regions (light pink column in Fig. 1b) but only 6.3% of FSTs was located in intron regions (dark pink column in Fig. 1b), significantly lower than 14.1% by Z test ($p = 0.0095$). The results suggest the preference for *Ds* elements to transpose into coding regions.

We then focused on the chromosome distribution of the 3029 FSTs. Our data showed that these FSTs were not evenly distributed on rice chromosomes (Supplementary Fig. 1). Generally, lower frequency of *Ds* insertions was observed around centromeres of all chromosomes. On the contrary, more density of *Ds* insertions was detected at the beginnings and ends of some chromosomes (Supplementary Fig. 1). For example, 23.3% of FSTs were located on the first 5-Mb region of chromosome 1, which occupied only 11.5% of the chromosome.

Screening for *Ds* insertion lines with abiotic stress tolerance or sensitivity and their tagged genes

Around 20,000 *Ds* insertion lines were subjected to various abiotic stresses including drought, high salinity, and cold stresses to evaluate their performance according to the description by Jiang et al. (2007). Totally, we have obtained 833 *Ds* insertion lines with changed responses to drought, high salinity or cold stress. Genomic DNA samples from a total of 300 lines were extracted from leaves for TAIL-PCR followed by sequencing to obtain *Ds* FSTs. Finally,

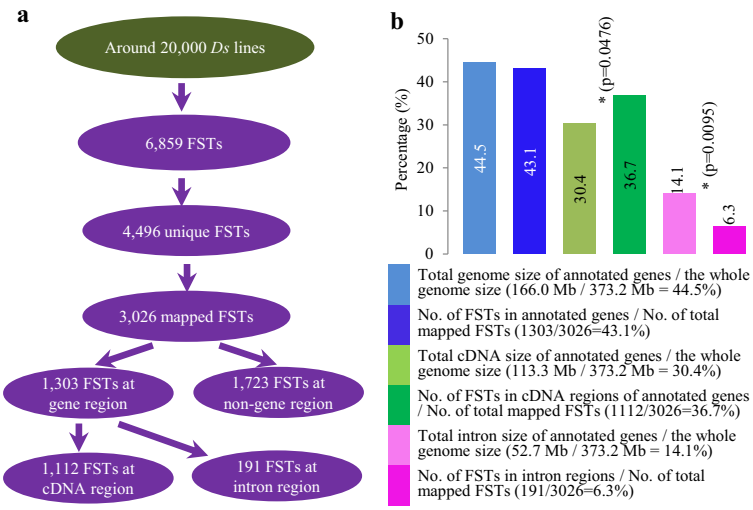


Fig. 1 Flanking sequence tags and their analysis. **a** Flow diagram to show FST filtering and their classification. A total of 3026 FSTs were mapped on the rice genome although 4496 unique FSTs were obtained by filtering a total of 6859 FSTs. Among 3026 mapped FSTs, 1303 FSTs were found in gene region including 1112 FSTs at coding region and 191 FSTs in intron region. The rest 1723 FSTs were at non-gene region. **b** Analysis of FST distribution on the rice genome. The chart shows the comparative frequencies of *Ds* insertion into different regions of the rice genome including gene and non-gene regions, coding and non-coding regions, and exon and intron regions

we have obtained FSTs from 202 lines (Fig. 2a). Sequencing analysis showed that some of these FSTs were from vector sequences and were not used for further analysis. Finally, a total of 165 rice genes were assigned with corresponding FSTs. Among them a total of 34 genes were annotated as "encoding expressed protein" and 19 genes encode transposable elements (Fig. 2a). The remaining 112 genes have been assigned with GO (The gene ontology consortium 2015) terms (Fig. 2a).

We further carried out the GSEA (Mootha et al. 2003). We detected a total of four over-represented GO terms including three GO terms from "Biological Process" and one GO term from "Molecular Function" (Fig. 2b). The three GO terms from "Biological Process" include GO:0009987 (cellular process), GO:0008152 (metabolic process), and GO:0050896 (response to stimulus). Thus, among these abiotic stress tolerant or sensitive lines, *Ds*-tagged genes showed over-representation in these biological processes. For example, among the *Ds*-tagged genes, 25% of them showed the functions in responses to stimulus while only 20.2% of the total annotated genes (with GO terms assigned) exhibited the functions (Fig. 2b). The over-represented GO term from "Molecular Function" is GO:0003824 (catalytic activity), suggesting the possible roles of catalytic activity in abiotic stress pathways.

Expression profile of 165 genes among 11 rice genotypes under either high salinity or drought stress

To better understand the possible biological functions and to explore the potential as molecular markers of the 165 *Ds*-tagged genes, these genes were subjected to expression analysis. We employed RNA-seq to survey their expression regulation. The rice variety Nipponbare shows sensitivity to both high salinity and drought stresses and was used as a control genotype. A total of five high salinity and five drought tolerant lines were selected for expression analysis. These five salinity tolerant lines are listed as PI279132, PI399813, PI400694, PI439048, and PI464588. The five drought tolerant lines are PI201839, PI224899, PI373139, PI388466, and PI584612. Among the 165 investigated *Ds*-tagged genes, 159 genes showed expression signal and their expression data were deposited in Supplementary Table 1. Among the 165 investigated genes, 56 genes (34%) showed up- or down-regulated by either high salinity or drought stress among 11 rice genotypes (Supplementary Fig. 2a and b). Generally, significantly higher percentages of genes were regulated by 250 mM NaCl when compared with those regulated by 30% PEG. Under 250 mM NaCl, 54 out of 165 genes were differentially expressed under this stress in a total of 6 rice genotypes. In Nipponbare, a total of 17 and 18

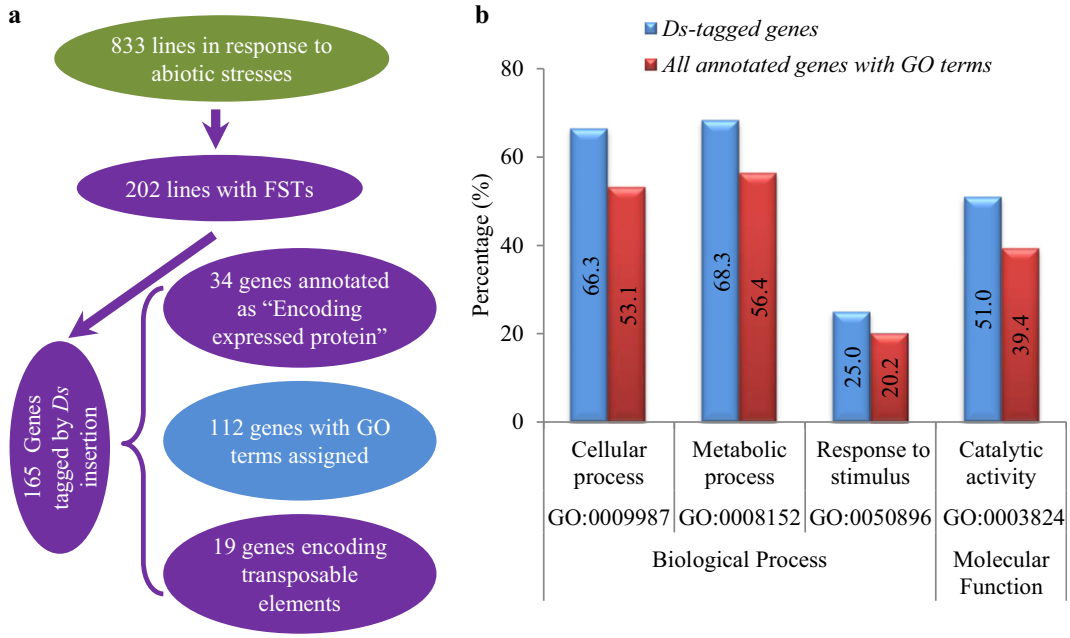


Fig. 2 *Ds* insertion lines showing tolerance or sensitivity to abiotic stresses and their FST analysis. **a** A total of 833 *Ds* insertion lines were identified with improved tolerance or showing more sensitivity to high salinity, drought, or cold stress. FSTs were obtained from 202 lines and a total of 165 genes were tagged including 34 genes encoding "expressed protein," 112 genes with GO terms assigned and 19 genes encoding transposable elements. **b** Gene ontology and gene set enrichment analysis of *Ds*-tagged genes. A total of 112 genes were assigned with GO terms, which were subjected to gene set enrichment analysis. Three GO terms including GO:0009987, GO:0008152, and GO:0050896 from Biological Process and one GO term GO:0003824 from Molecular Function show the difference between *Ds*-tagged genes and all annotated genes with GO terms in the rice genome

genes were up- and down-regulated, respectively (Supplementary Fig. 2a). Compared with Nipponbare, less percentages of genes were up- or down-regulated by 250 mM NaCl in five salinity tolerant lines (Supplementary Fig. 2a). For example, in PI439048, 12 and 14 genes (17%) were up- and down-regulated by 250 mM NaCl, respectively. Under 30% PEG stress, only 22 out of 156 genes showed up- or down-regulation in their expression (Supplementary Fig. 2b). A total of 30 genes (labeled by superscript "m" in Supplementary Fig. 2) were regulated only by high salinity stress. Three genes (*04g46190*, *05g02020*, and *08g44200*, labeled by superscript "n" in Supplementary Fig. 2) were up- or down-regulated only by 30% PEG. A total of 12 genes (labeled by blue fonts) were differentially expressed in all high salinity tolerant rice lines and two genes (*03g14590* and *08g33140*, labeled by green fonts) were up-regulated in all drought tolerant lines. Interestingly, we have detected only one gene *03g18130* (labeled by red fonts), whose expression was up-regulated by both 250 mM NaCl and 30% PEG in all tested rice genotypes (Supplementary Fig. 2). The gene encodes asparagine synthetase and the evidence from other species shows its involvement in high salinity or drought response (Wang et al. 2005; Maaroufi-Dguimi et al. 2011).

High percentages of *Ds*-tagged genes were up- or down-regulated by various hormone treatments

A total of 165 *Ds*-tagged genes were subjected to expression profiling using microarray data as described in the "Materials and methods" section. Totally, we have analyzed the expression regulation of these genes under six different hormone treatments including ABA, BR, GA, IAA, JA, and CK. Among the 165 genes, 79 genes showed up- or down-regulation by at least one of six hormone treatments (Supplementary Fig. 3). Among the detected 79 genes, most of them were differentially regulated by either ABA or JA. In roots, the expression level of a total of 19 genes was increased under ABA treatment; other 19 genes were down-regulated by the hormone treatment (Supplementary Fig. 3). On the contrary, 15 and 10 genes were up- and down-regulated by ABA treatment in the shoots (Supplementary Fig. 3). Upon JA treatment, much more numbers of genes were

differentially expressed when compared with ABA treatment. In roots, 25 and 33 genes were up- and down-regulated by JA treatment, respectively; in shoots, the numbers of up- and down-regulated genes were reduced to 17 and 25, respectively (Supplementary Fig. 3). Besides ABA and JA, these *Ds*-tagged genes were also differentially regulated by other hormones including BR, GA, IAA, and CK. However, compared with both ABA and JA, much lower percentages of these *Ds*-tagged genes were regulated by these four different hormones. For example, only five genes were up- or down-regulated by GA treatment.

Under the same hormone treatment, expression regulation might show difference between roots and shoots. Generally, both roots and shoots showed similar expression regulation for a gene under a same hormone treatment. For example, the gene *01g48680* was up-regulated by ABA in both roots and shoots (Supplementary Fig. 3). The second case is that a gene is up- or down-regulated by a hormone in roots but the gene shows no difference in shoots under the similar condition. For example, the gene *01g38229* was down-regulated by ABA in roots but no significant difference was observed in shoots under the similar condition (Supplementary Fig. 3). The third case is that both roots and shoots show opposed expression regulation. For instance, the gene *01g27170* was up-regulated by ABA in roots but was down-regulated in shoots (Supplementary Fig. 3). These results might imply the difference in their biological functions of these genes between roots and shoots.

Co-regulation of *Ds*-tagged genes by both abiotic stresses and hormone treatments

We have carried out the expression regulation of *Ds*-tagged genes by 250 mM NaCl and 30% PEG and revealed high percentages of genes showing differential expression under these conditions. Here, we further surveyed if there were co-regulations in some of these genes by both abiotic stresses and hormone treatments. As the highest percentages of *Ds*-tagged genes were regulated by either ABA or JA in their expression, these two hormones were selected for surveying their interactions with abiotic stresses. In Nipponbare, we detected 8 genes showing regulation by both NaCl and PEG, 27 genes regulated by only NaCl, and 3 genes regulated only by PEG (Supplementary Figs. 2 and 3a). Among the eight NaCl/PEG regulated genes, six of them were also up- or down-regulated by ABA and JA including *01g38229*, *01g55500*, *03g14590*, *03g18130*, *07g37550*, and *08g33140*; the gene *03g16860* was down-regulated by ABA, and the rest one *01g13760* was not regulated by any hormones (Fig. 3a). Among the three PEG-regulated genes, two of them (*04g52190* and *08g44200*) were also regulated by ABA and JA; the rest *04g46190* was ABA and JA independent (Fig. 3a). For the 27 NACl-regulated genes, only 7 genes *01g58220*, *02g43550*, *03g20790*, *03g49350*, *07g29290*, *09g30180*, and *10g42439* were not regulated by either ABA or JA (Fig. 3a). The remaining 20 genes were all up- or down-regulated by ABA or JA or both of them (Fig. 3a). Thus, 29 out of 38 NaCl/PEG-regulated genes (76%) were also regulated by ABA or JA treatment, suggesting that high percentages of genes might play roles in abiotic stresses by interacting with hormone signaling.

Although up to 76% of NaCl/PEG-regulated genes were also regulated by ABA/JA, there are many other *Ds*-tagged genes were not regulated by NaCl/PEG but regulated by ABA/JA. These genes might not be directly related to these abiotic stresses. We have detected a total of 49 genes, whose expression was regulated by only by ABA or JA but not by NaCl or PEG (Fig. 3b). Among these genes, 22 of them were up- or down-regulated by both ABA and JA in either roots or shoots or both of these two tissues. A total of 23 genes were regulated only by JA, and the rest 4 genes were regulated only by ABA. Compared with roots, less genes were up- or down-regulated by ABA/JA in shoots and we detected only 10 such genes out of 49 genes. Thus, roots are the important tissue for ABA/JA signaling in plants and play crucial roles in abiotic stresses.

Single nucleotide polymorphisms (SNPs) of 165 *Ds*-tagged genes in five high salinity and five drought tolerant rice lines

To explore the molecular polymorphisms between high salinity/drought tolerant and sensitive lines in these 165 *Ds*-tagged genes, SNPs were surveyed by RNA-Seq. Based on our RNA-Seq data, orthologous genes have been found in all ten rice lines when compared with the reference rice line Nipponbare for all 165 *Ds*-tagged genes. Thus, SNPs in each gene were detected by comparing with the reference genome Nipponbare. Among the 165 *Ds*-tagged genes, 102 genes (62%) were detected with SNPs (Fig. 4a–c). The details on gene locus names, SNP positions, and changed bases in all of ten

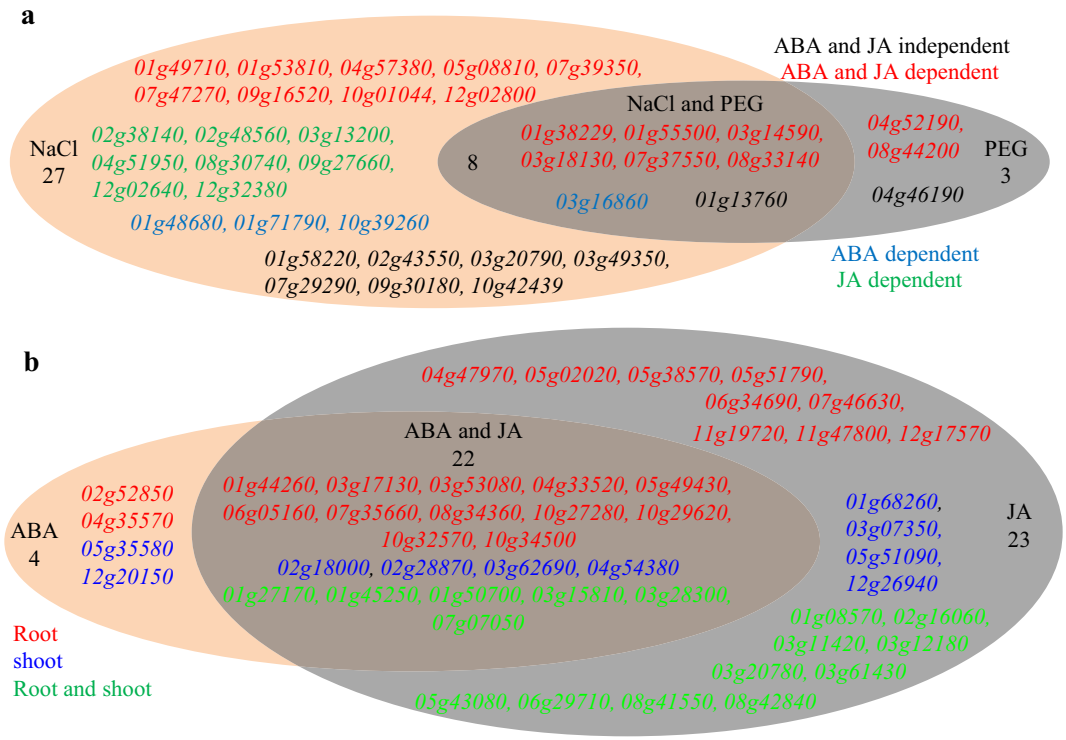


Fig. 3 *Ds*-tagged genes showing expression regulation under abiotic stresses and hormone treatments in Nipponbare. **a** Venn diagram showing interaction of abiotic stress-regulated genes with hormone signaling. Black, red, blue, and green fonts of locus names indicate these genes with ABA and JA independent, ABA and JA dependent, ABA dependent, JA dependent expression patterns, respectively. **b** Genes showing no differential expression under abiotic stresses but regulated by ABA and/or JA. Red, blue, and green fonts of locus names indicate these genes showing significant expression regulation in root, shoot, and root and shoot, respectively

rice lines are listed in Supplementary Table 2. Some of these genes have only one SNP in some rice lines. For example, *01g50700*, *01g63840*, and *02g16060* have only one SNP in the rice line PI224899 (Supplementary Table 2). Most of these genes contain multiple SNPs in each rice lines (Fig. 4a–c). A total of 251 SNPs were detected in the gene *06g29710* among the ten rice lines (Fig. 4b). Generally, different genes showed different numbers of SNPs among different rice lines. For each gene, some rice lines showed the different numbers of SNPs but others showed the same SNP numbers. For example, for the gene *01g13370*, 5, 6, and 8 SNPs were detected in the lines PI373139, PI399813, and PI439048, respectively; 7 SNPs each were detected in the lines PI279132, PI464588, and PI201839; no SNP was detected in the rest 4 rice lines (Supplementary Table 2). Thus, it is difficult to differentiate different rice lines using the SNP data from only one gene. However, it is possible to differentiate any genotype from the ten lines by using the combination of selected two genes. For example, both *12g17570* and *12g20150* can be used for the purpose by analyzing their SNPs among the ten rice lines. Generally, based on the SNP analysis, any genotype could be differentiated from others by using the SNP data from any 6 out of 102 genes. It means that any six genes could be used to differentiate a genotype from the rest. On the other hand, we could not identify any SNPs that showed the significant co-relationship with either high salinity or drought stresses. In addition, only SNPs in coding regions were identified and the large-effect SNPs due to exon-intron splicing were not identified. Furthermore, no large-effect SNPs due to the change of start or stop codons was identified in this study.

Insertions and deletions (indels) of 165 *Ds*-tagged genes in five high salinity and five drought tolerant rice lines

Indels consist of 1–10 bp of insertions or deletions. We used Nipponbare as the reference genome to detect

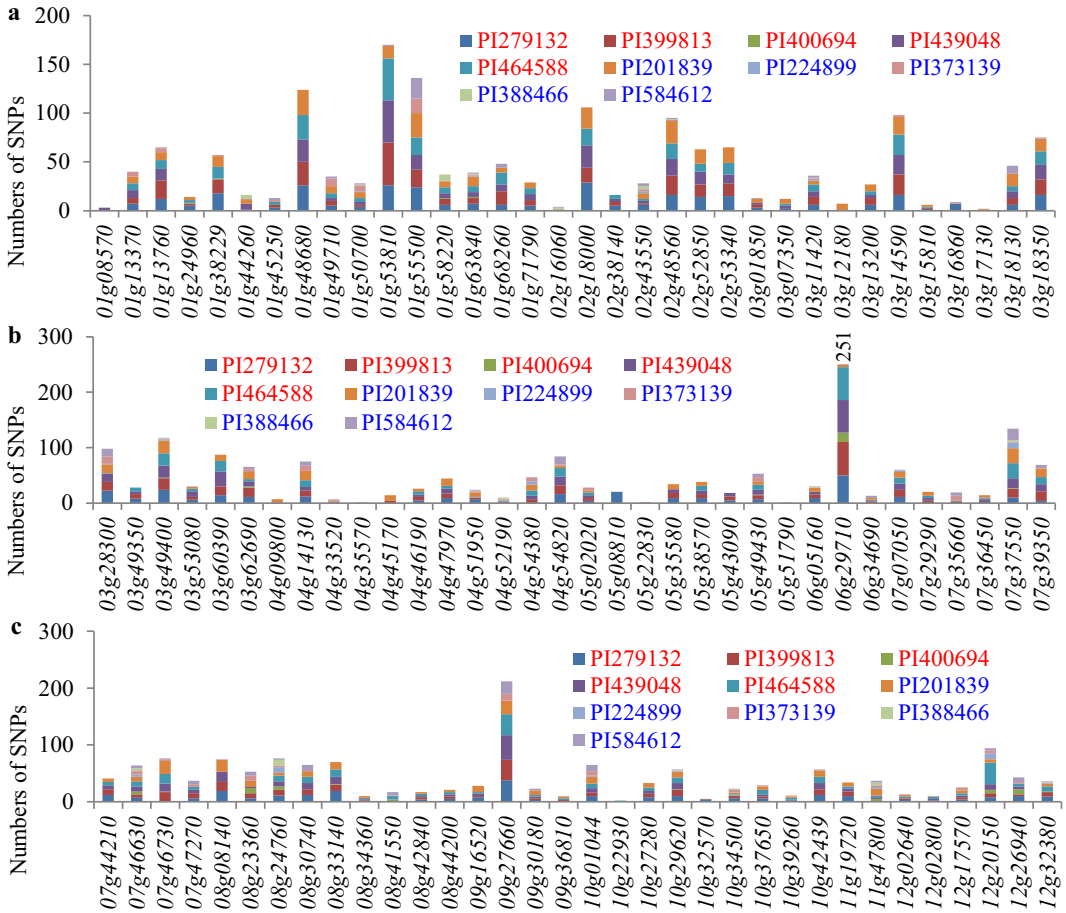


Fig. 4 Detection of single nucleotide polymorphisms of *Ds*-tagged genes among five high salinity and five drought tolerant rice lines. **a**–**c** Detectable SNPs among ten rice lines in *Ds*-tagged genes 1–34, 35–68, and 69–102, respectively. SNPs were detected by the program GATK (v3.4-0, McKenna et al. 2010) as described in the "Materials and methods" section. A total of 102 genes out of 165 *Ds*-tagged genes were detected with SNPs in either one or more genotypes of ten rice lines. Numbers of SNPs indicate the cumulative SNPs from all rice lines. Rice lines labeled with red and blue fonts indicate that these lines are high salinity and drought tolerant lines, respectively

indels of 165 *Ds*-tagged genes among ten rice lines including five high salinity and five drought tolerant lines. Among the 165 *Ds*-tagged genes, 39 of them (24%) contain indels (Fig. 5a and Supplementary Table 3). Different from SNPs, less percentage of genes have been suffered from indels. Similar to SNPs, among the indel-containing genes, different genes have different numbers of indels among ten rice genotypes (Fig. 5). Some genes may have only one indel. For example, for the gene *02g38140*, only one indel from A to AG was detected in the rice line PI279132 (Supplementary Table 3) and the indel was not detected in any other rice lines. Some genes have multiple indels that were present in multiple rice lines. For example, the gene *03g28300* have 19 indels, which were presented in six rice lines including PI279132, PI399813, PI439048, PI201839, PI373139, and PI584612 (Fig. 5 and Supplementary Table 3). Similarly, no indels were detected, which shows significant co-relationship with either high salinity or drought stress tolerance.

One type of indels consists of insertions and deletions with multiples of 3 bp and produce no frame-shift mutations during protein translation. Another type of indels are not with multiples of 3 bp insertions and deletions, and thus, produce frame-shift mutations, which were designated as the large-effect indels. We have detected 29 genes with large-effect indels from 39 indel-containing genes (Fig. 5b and Supplementary Table 4). Among the 29 genes, indels from 4 genes (labeled with red fonts in Fig. 5b) were detected only in one or two

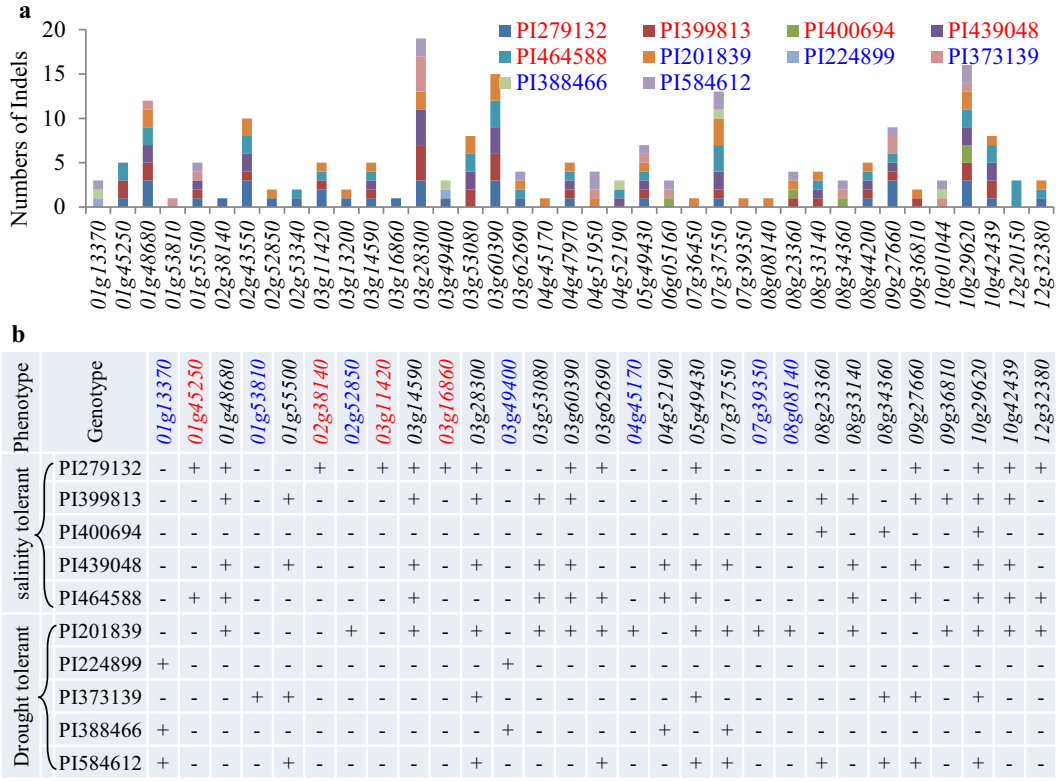


Fig. 5 Detection of 1–10 bp insertions or deletions of *Ds*-tagged genes among 5 high salinity and 5 drought tolerant rice lines. **a** Indels were detected in 39 out of 165 *Ds*-tagged genes. Indels were detected by the program GATK (v3.4-0, McKenna et al. 2010) as described in the "Materials and methods" section. Numbers of indels indicate the cumulative indels from all rice lines. **b** Indels with large-effect detected among ten rice lines and their distribution in these lines. Rice lines labeled with red and blue fonts indicate that these lines are high salinity and drought tolerant lines, respectively. A total of 29 out of 39 indels-containing genes were detected with large-effect indels. Blue and red locus name in **b** indicates that the corresponding gene was suffered from frame-shift mutation only in drought and high salinity stress tolerant lines, respectively

high salinity tolerant rice lines. For other seven genes (labeled with blue fonts in Fig. 5b), the large-effect indels were detected only in drought tolerant rice lines. For the remaining 18 genes, the large-effect indels were detected in both high salinity and drought tolerant lines (Fig. 5b). However, no co-relationship between the large-effect indels and stress tolerance has been detected.

Discussion

Ds mutagenesis as a tool to generate mutants for functional genomics of abiotic stress tolerance in rice

The maize *Ac/Ds* transposons have been successfully used for insertional mutagenesis in Arabidopsis, rice, and many other species (Ramachandran and Sundaresan 2001; Hirochika et al. 2004; Lazarow and Lütticke 2009; Carter et al. 2013; Fujimoto et al. 2016). Compared with T-DNA insertion lines, less genes were functionally characterized in rice by *Ds* insertion lines (Jiang and Ramachandran 2010) although more than 20,000 *Ds* FSTs were available in various rice scientific research communities (Krishnan et al. 2009). Only a few cases were reported on the application of *Ds* insertion mutagenesis on the functional genomics of abiotic stress tolerance in rice and other plants. Most examples were from Arabidopsis. Mutant analysis of *Ds* insertion lines showed that AtMYC2 functions as a transcriptional activator in ABA-inducible gene expression under drought stress in Arabidopsis (Abe et al. 2003). Phenotypic and functional characterization of an Arabidopsis *Ds* insertion line showed the role of putrescine in high salinity stress (Urano et al. 2004). A *Ds albino or pale-green* (*apg*) mutant analysis showed that

APG6 play a role in heat-stress response in Arabidopsis chloroplasts (Myouga et al. 2006). In rice, only one abiotic stress-related gene was functionally characterized by *Ds* insertion line. The gene was named as *OsCIPK31*, encoding a CBL-interacting protein kinase (Piao et al. 2010). Their detailed analysis showed that *OsCIPK31* played roles in germination and seedling growth under abiotic stress conditions (Piao et al. 2010). Generally, very limited data were available in the application of *Ds* insertion on the functional genomics of abiotic stresses. In our study, 833 lines were obtained to be responsive to abiotic stresses by screening around 20,000 *Ds* insertion lines. Among them, 202 lines have their FSTs which were inserted into gene regions. In some of these lines, *Ds* was inserted into a same gene region. As a result, a total of 165 genes were tagged by *Ds* insertion. We further investigated their gene expression profiles under normal and abiotic stress as well as various hormone treatments. Our analysis provided additional information of these genes for interested scientists to further carry out their functional characterization.

Cross-talks among hormones and abiotic stresses in *Ds*-tagged rice lines

Abiotic stress-responsive genes were regulated by ABA-dependent and ABA-independent pathways (Yamaguchi-Shinozaki and Shinozaki 2006; Yoshida et al. 2014). By using rice Affymetrix chips consisting of 49,824 transcripts from 55,515 probes, Garg et al. (2012) identified 2086 up-regulated and 1549 down-regulated genes; thus, up to 7.3% of the rice genes were differentially expressed under ABA treatment. In this study, we identified 165 *Ds*-tagged genes and their corresponding *Ds* insertion lines showed significant difference in their response to drought, high salinity or cold stress. GO term enrichment analysis showed that significantly higher ratio of genes were assigned to GO terms related to "response to stimulus" (Fig. 2b), suggesting their roles in abiotic stress pathways. Expression analysis showed that 47 of them (28.5%) were up- or down-regulated by ABA treatment in either shoots or roots (Supplementary Fig. 3). Thus, higher ratio of abiotic stress-related genes was regulated by ABA. These genes encode various proteins such as cation transporter (*01g27170*), dehydrin (*01g50700*), transcription factor (*08g34360*), protein kinase (*10g29620*), and so on. On the other hand, we have also detected 22 genes, which were regulated by other hormones but not by ABA treatment. Some of these genes may also play roles in abiotic stress pathways as at least eight ABA-independent genes were regulated by NaCl or PEG (Fig. 3a). Some of these genes might play roles by interacting with ABA-dependent genes as there are cross-talks between ABA-dependent and ABA-independent genes (Roychoudhury et al. 2013).

Interestingly, compared with ABA-regulated genes, we have detected higher ratio of genes with JA-dependent expression pattern. A total of 70 out of 165 *Ds*-tagged genes (42.4%) were up- or down-regulated by JA treatment (Supplementary Fig. 3). The JA signal pathway is directly related to biotic stress (Turner et al. 2002). Some of these genes encode resistance gene analog RGA (*02g16060*, *02g18000*), ABC transporter (*08g30740*), leucine rich repeat protein (*08g42840*), and various transcription factors. Studies showed that RGAs functioned in disease resistance (Sekhwal et al. 2015). ABC transporters play roles in disease resistance and abiotic stress responses (Rea 2007; Kang et al. 2010; Kuromori et al. 2010). There exists crosstalk between abiotic and biotic stress response (Fujita et al. 2006). Thus, these JA-regulated genes may also play roles in biotic stress signaling pathways and their corresponding *Ds* insertion lines may also show different responses to some of biotic stresses.

Towards variety development with improved tolerance to high salinity and/or drought tolerance

One of the final purposes to explore the mechanisms of abiotic stress tolerance is to develop varieties that exhibit improved tolerance to high salinity and/or drought stresses. One of the breeding prerequisite is that we have breeding parent lines or germplasms showing better tolerance to these abiotic stresses. In fact, based on the collection from National Plant Germplasm System in the US Department of Agriculture Agricultural Research Service (https://www.ars-grin.gov/npgs/index.html), many tolerant rice lines are available for the breeding purpose. For example, in this study, we have collected five high salinity and five drought tolerant rice lines from the system, which form the basis to provide gene donors to develop the higher tolerant rice lines. Thus, the key to develop the tolerant rice lines is how to identify stress-related genes or their molecular markers. By screening *Ds* insertion lines, we have obtained 833 *Ds* insertion lines with differentiated

sensitivity to high salinity, drought, or cold stress. We have also obtained 165 *Ds*-tagged genes from these 833 lines. We may further functionally characterize these 165 *Ds*-tagged genes and explore their potential as donor genes for further improving abiotic stress tolerance by over-expression or gene silencing or gene knockdown /knockout through various strategies including RNAi technique (Kusaba 2004) or CRISP-Cas 9 (Ma et al. 2015), etc.

On the other hand, we have explored SNPs and indels among these 10 NaCl or drought tolerant lines. Our data showed that SNPs or indels are universally presented for the 165 *Ds*-tagged genes in all of the ten rice lines when compared with the Nipponbare sequence (Figs. 4 and 5). The results make it possible for us to utilize these SNPs and indels as molecular markers for breeding selection. As long as the biological functions of a *Ds*-tagged gene is verified, the gene itself or its corresponding SNPs or indels will be used as molecular markers. These SNPS or indels have also provided the basis for pyramiding breeding when multiple genes are required for the improved tolerance. Generally, our data may provide useful information for both characterizations of gene functions and molecular marker-assisted selection.

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Compliance with ethical standards This research does not involve human participants or animals.

Conflict of interest The authors declare that they have no competing interests.

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