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Comprehensive transcriptomics and proteomics analyses of *rice stripe virus*-resistant transgenic rice

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Stable transgenic rice line (named KRSV-1) with strong resistance against *rice stripe virus* was generated using the gene sequence of disease-specific protein by RNA interference. Comprehensive safety assessment of transgenic plants has turned into a significant field of genetic modification food safety. In this study, a safety assessment of KRSV-1 was carried out in a stepwise approach. The molecular analysis exhibited that KRSV-1 harbored one copy number of transgene, which was integrated into the intergenic non-coding region of chromosome 2 associated with inter-chromosomal translocations of 1.6-kb segments of chromosome 8. Then, transcriptomics and proteomics analyses were carried out to detect the unintended effects as a result of the integration of the transgene. Although 650 dramatically differentially expressed genes (DDEGs) and 357 differentially expressed proteins were detected between KRSV-1 and wild-type (WT) by transcriptomics and proteomics analyses, no harmful members in the form of toxic proteins and allergens were observed. Encouragingly, the nutritional compositions of seeds from KRSV-1 were comparable with WT seeds. The results of this entire study of molecular analysis, transcriptome and proteome profile of KRSV-1 revealed that no detrimental changes in the form of toxic proteins and allergens were detected in the transgenic rice line due to the integration of the transgene.

Keywords. Biosafety assessment; *Oryza sativa*; proteomics; *rice stripe virus*; transgenic rice; transcriptomics; virus resistance

1. Introduction

Oryza sativa L. is a staple food for more than half of the global population, which plays an extremely important role in food production and security (Zhou *et al.* 2017). However, rice diseases caused by several pathogens are major obstacles for achieving the optimal yield (Khush 2005; Ke *et al.* 2017). Viral diseases of rice significantly affect the yield of this crop in much of the world's rice-growing areas (Ma *et al.* 2011). *Rice stripe virus* (RSV) belongs to the genus *Tenuivirus*, which is transmitted by a small brown planthopper (*Laodelphax striatellus*, Fallen) in circulative–propagative and transovarial manners. It causes rice stripe disease, which is one of the most disreputable epidemics in temperate and subtropical regions (Zhao *et al.* 2016). Typical symptoms of rice infected by RSV include discontinuous chlorotic stripes, mottled leaves and withered inner leaves with more severe infection (Shi *et al.* 2016). Eventually, the excess parasitic load causes growth termination and plant death, leading to considerable yield loss or even total yield failure.

Until now, only few RSV resistance genes have been functionally characterized. An RSV-resistance gene, *Stvb-i*, from *Indica* rice that was introgressed into several *Japonica* rice cultivars provided stable resistance to RSV (Hayano-Saito *et al.* 1998); *STV11*, another RSV-resistant gene encoded a sulfotransferase, conferring durable resistance to RSV (Wang *et al.* 2014). However, such conventional

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breeding is extremely difficult and time-consuming due to the emergence of new RSV rice line and limited knowledge on RSV-resistant genes (Park *et al.* 2012; Wang *et al.* 2014). RNA interference (RNAi) is a viral defense mechanism that can offer a viable method to produce RSV-resistant plants (Shimizu *et al.* 2011). Genetic engineering of the host plants with virus-derived transgenes can offer sequence-specific RNA to degrade invading viral RNA through a process known as RNA-mediated viral resistance (Vazquez Rovere *et al.* 2002).

Genetically modified (GM) plants or transgenic plants were first commercialized in 1996 and had the potential to solve the world's hunger and malnutrition problems, increase the yield and reduce the use of synthetic chemical pesticides (Bawa and Anilakumar 2013). However, the public raised extensive concerns about the potential risk on human and animal health and the environment resulting from genetic modifications. Among them, food allergy is an important health issue (Cao *et al.* 2012). Plants have evolved to synthesize various noxious or allergens to cope with unfavorable environments, among which, a large group of toxic proteins and allergens may play critical roles in plant growth development or defense against predators and microbes but may induce severe alterations in the body tissues of both humans and animals (Russell *et al.* 2008; Wakasa *et al.* 2013; Dang and Van Damme 2015). A substantial proportion (3.7%) of adults and approximately 6–8% of children in the USA suffer from allergic disease, particularly food allergy (Cao *et al.* 2012). Novel foods such as those derived from the GM process have been the focus of public concerns about the likelihood of adverse health effects, including allergenicity. Whether the encoding product of a novel gene in GM plants will have the potential to induce *de novo* sensitization or to elicit hypersensitivity reactions in already-sensitized known allergens remains unknown (Cao *et al.* 2012). Therefore, the requirements for the safety assessment of GM plant allergenicity are vital for the eventual commercialization of transgenic crops.

Biosafety assessment is composed of extensive molecular analysis and comprehensive unintended effect evaluation (Kok *et al.* 2014). Development of specific and efficient detection methods is not only useful for breeding programs but also of important for biosafety management to ensure food and environmental safety (Holst-Jensen *et al.* 2012; Fraiture *et al.* 2015). Traditionally, molecular analysis involves the examination of transgene copy number, T-DNA integration sites and transgene expression. The evaluation of unintended effects that arises from genetic modifications is another indispensable aspect of transgenic crop safety assessment, which is widely performed by using omics techniques, which are made up of different biological levels, such as transcriptomics, proteomics and metabolomics profiling to reveal the corresponding levels of divergence and unintended effects, including the contents of toxic protein and allergens (Sanchez Perez *et al.* 2009; Zhao and Li 2013; Wang *et al.* 2015; Sun *et al.* 2016).

Research on the biosafety assessment of transgenic plants has attracted much interest and numerous studies have been reported. Most of them specializes in carrying out the molecular analysis of the foreign T-DNA integration pattern (Zhai *et al.* 2004) or performing single transcriptomics, proteomics or metabolomics analysis of unintended effects resulting from genetic modification processes (Rischer and Oksman-Caldentey 2006; Ren *et al.* 2009; Lambirth *et al.* 2015; Mishra *et al.* 2017). The comprehensive application of various approaches is relatively few in previous studies. More sophisticated methods are urgently needed to evaluate the potential biosafety risk of the unintended effects of GM plants.

In the present study, stable transgenic rice line contained inverted repeat sequence of disease-specific protein (*IRSP*) of RSV had enhanced resistance to RSV diseases. Molecular analysis, i.e. the transgene copy number, and insertion site analysis were performed by Southern blot hybridization and high-efficient thermal asymmetric interlaced polymerase chain reaction (hiTAIL-PCR), respectively. Moreover, targeted techniques such as nutritional composition assessment and non-targeted techniques including transcriptome and proteome were comprehensively analyzed for unintended effects. These data revealed that the stable transgenic rice line KRSV-1 was competitive with non-transgenic cultivars, which had no significant differences in terms of nutritional ingredients compared with wild-type (WT) plants, and toxic proteins or allergens were not observed in KRSV-1 through transcriptomics and proteomics analyses.

2. Materials and methods

2.1 *Plant materials and growth conditions*

Rice variety, Xiangjing9407, was used for generating transgenic lines. Transgenic rice with strong resistance against RSV (containing a partially inverted repeat sequence of the *SP* gene, *IRSP*) was developed by RNAi (Ma *et al.* 2011). Transgenic plant generation and vector sequence were provided in a previous paper (Ma *et al.* 2011). The self-fertilized homozygous T_6 progeny of the transgenic rice line SP-7 (renamed KRSV-1), which contained a p1300 expression vector, was used as the material for further analyses. Plant materials were grown in the field at the experimental stations of Shandong Agricultural University.

2.2 *Analysis of RSV resistance of transgenic plants*

For RSV resistance analysis, the T_6 transgenic rice line and WT rice were sown separately into 1 L beakers with 25 seeds per cup. For viral infection, plants were exposed to the viruliferous or virus-free insects of *L. striatellus* Fallen for 72 h upon reaching the 5–10 leaf stage. Then, the insects were removed, and the plants were grown in experimental

stations. We scored the development of symptoms from the 7th day after inoculation once every 3 days and six times total as described in the previous study (Ma *et al.* 2011).

2.3 *Southern blot and identification of T-DNA insertion site*

The total DNA of the RSV-resistant transgenic plants and WT was extracted using the phenol method (Ma *et al.* 2011). Three grams of RSV-resistant transgenic plants and WT plants were ground into fine powder and homogenized with the extraction buffer, hexadecyl trimethyl ammonium bromide (CTAB), and then purified with phenol–chloroform–isoamyl alcohol. Then, the extract was centrifuged at 7500×*g* for 10 min, and the supernatant was transferred into a 2 mL centrifuge tube. Two volumes of cold absolute ethanol were added, followed by incubation at −20°C for at least 2 h. The dissolved DNA underwent restriction enzyme digestion in a volume of 200 μL in the presence of 40 ng genomic DNA, 20 μL 10× buffer and 8 μL restriction enzyme (Ma *et al.* 2011). Probe preparation, hybridization and chemiluminescence detection of total DNA were performed according to the manufacturer's instructions (DIG Southern Starter Kit, Roche, Switzerland). To determine the T-DNA insertion site, we adopted the standard CTAB methods to extract genomic DNA and used the hiTAIL-PCR approach to determine the sequences flanking T-DNA insertions (Liu *et al.* 1995; Kneidinger *et al.* 2001). Random primers and nested insertion-specific primers were designed. Specific primers KRSV-1 LB-0b, LB-1b and LB-2b were used to amplify the sequence upstream of the 5′ region, and primers KRSV-1 RB-0b, RB-1b and RB-2b were used to amplify the sequence downstream of the 3′ region. Arbitrary degenerate primers LAD1-1, LAD1-2, LAD1-3, LAD1-4 and AC1 were designed, and the detailed procedures of hiTAIL-PCR were based on the original publication (Liu and Chen 2007). For the hiTAIL-PCR amplification of genomic DNA flanking the T-DNA insertion sites, the amplified right and left T-DNA border sequences and adjacent sequences from rice were used to search the GenBank of the NCBI genome database (*https://www.ncbi.nlm.nih.gov/*). Blast analysis was performed to identify the T-DNA insertion site. The sequences were aligned to the genome sequence to identify the genomic and chromosome positions of the insertions.

Then, genomic DNA from the KRSV-1 stable transgenic strains was used for genome walking by using the Genomewalker^{TM} Universal kit (Clonetech, Mountain View, USA) to verify the accuracy of the insertion site. Gene-specific primers (GSP1 and GSP2) for genome walking and primers for genomic DNA amplification were designed, and PCR was carried out for genomic DNA as per the manufacturer's instructions (100 ng genomic DNA, 1.5 mM $MgCl_2$, 200 μM dNTP, 200 nm each primer and 1 unit of *Taq* DNA polymerase, total 25 μL) (Siebert *et al.* 1995). The flanking sequences were then subjected to the BLAST procedure to search public DNA databases.

2.4 *RNA extraction and quantitative real-time PCR (qRT-PCR)*

Seedling samples (0.1 g) were extracted with TRIzol reagent (Invitrogen, USA) according to the manufacturer's instructions. A concentration of 2 μg total RNA was reverse-transcribed with the FastQuant RT kit (TIANGEN, China) by using the FQ-RT Primer Mix according to the manufacturer's instructions. qRT-PCR reactions were performed using the SYBR Green Real-Time PCR Master Mix (Vazyme Biotech, China) and a CFX96TM Real-Time PCR Detection System (Bio-Rad, USA) (Xu *et al.* 2018). Relative transcript levels were calculated using the $2^{-\Delta\Delta C(t)}$ method with the *Osactin* transcript as the internal standard. Each data set was derived from three biological repeats. The primers are listed in supplementary table 1.

2.5 *Analysis of transcriptomics data*

For each sample, three biological replicates were prepared. For cDNA library construction, total RNA was extracted from seedling samples of transgenic plants and WT and then processed using an RNA Sample Preparation Kit (Bioteke, China, RP3322). For the RNA-Seq experiment, ∼10 mg of total RNA was subjected to enrichment of the poly (A)-tailed mRNAs with poly (T) oligo-attached magnetic beads. Clean tags were obtained by filtering the adaptor sequences and removing low-quality sequences containing ambiguous bases. Sequencing was carried out on each library to generate 100-bp paired-end reads for transcriptome sequencing on an Illumina High-Seq 2000 platform by a commercial service provider (San Diego, CA, USA). The expression level of each gene was estimated based on the frequency of clean tags and then normalized to number of transcripts per million clean tags (TPM), which is a standard method and is extensively used in differentially expressed gene (DEG) analysis (Morrissy *et al.* 2009). TPM indicates the reads per kilobase of transcripts per million of sequenced reads. A gene ontology (GO) term was assigned to each transcript based on the GO annotations for biological processes in The Rice Annotation Project Database (*http://rapdb.dna.affrc.go.jp*).

2.6 *Protein extraction and SDS-PAGE electrophoresis*

Rice seed proteins were extracted from the germinating seeds by the acetone precipitation method as per the details given in the previous study (Han *et al.* 2013). Briefly, 2 g of germinating seeds were ground into fine powder homogenized with extraction buffer (20 mM Tris/HCl, pH 7.5, 250 mM sucrose, 10 mM ethylene bis (oxyethylene nitrilo) tetra-

acetic acid (EGTA), 1 mM protease inhibitor cocktail, 1 mM phenylmethanesulfonyl fluoride (PMSF), 1 mM DL-Dithiothreitol (DTT) and 1% Triton X-100), and placed on ice for 10 min. Then, the extract produced was centrifuged at 15,000×*g* for 20 min, and the supernatant was transferred into a 2 mL centrifuge tube. Three volumes of cold acetone were added, followed by incubation at −20°C for at least 2 h. The supernatant was then centrifuged, and the pellets were washed with cold acetone thrice. The pellets were vacuum-dried and dissolved in H_2O for SDS-PAGE electrophoresis (30% Acr-Bis, 1.5 M Tris-HCl, 10% SDS, 10% ammonium persulfate (APS), N,N,N',N'-Tetramethylethylenediamine (TEMED) and constant current: 14 mA, 3–4 h).

2.7 *Analysis of the proteomics data by using the SWATH method*

For each sample, three biological replicates were prepared. Total proteins were extracted from seeds of KRSV-1 and WT plants through the acetone precipitation method. Then, proteome was performed at the Beijing Proteome Research Center. A Triple TOF 5600 mass spectrometer (SCIEX, USA) coupled to an Eksigent NanoLC-2DPlus with a nanoFlex cHiPLC system equipped with a 10-μm SilicaTip emitter (New Objective, Woburn, MA) was used for protein analysis. The MS data of the sample were searched using ProteinPilot 4.5 to generate the SWATH library, which contained information about the targeted proteins, peptides and their fragment ions. The generated library and the SWATH data files were together loaded into the PeakView (version 1.1.1) SWATH Processing Micro App (version 1.0, SCIEX, USA) for SWATH data-processing, which has been described previously (Zhang *et al.* 2016). A total of 1888 proteins from transgenic and WT plant samples were detected, and 357 differentially expressed proteins (DEPs) showing fold changes with $P<0.05$ were considered in the following study.

2.8 *Analysis of essential nutrient components and poisonous heavy metal ions*

Essential nutrient components and poisonous heavy metal ions were determined and analyzed with the help of Shandong Entry–Exit Inspection and Quarantine Bureau located in Qingdao, China.

Quantification analysis was conducted on heavy metal ions, such as Pb, Cd, Hg and Mn. Stable transgenic rice strain seeds (~1.0 g) were ground by high-speed tissue-grinding machine to powder, and the quickly collected materials were added to MARS Xpress (Microwave Accelerated Reaction System, CEM, USA) for microwave digestion for approximately 120 min. Finally, quantitative analysis was carried out by using ICP-MS 7500ce (Inductively Coupled Plasma Mass Spectrometry, Agilent, USA). The working parameters were the following: radio frequency power, 1500 W; flow rate of carrier gas, 0.97 L/min; integration time, 0.3 s; atomizing chamber temperature, 2°C; flow rate of the inert gas, 3.5 mL/min; $^{156}CeO^{+}/^{140}Ce^{+}$, 0.25%; double charge $^{70}Ce^{2+}/^{140}Ce^{+}$, 0.56%.

The entire proximate components such as the total protein, amino acid, carbohydrate and vitamin contents of KRSV-1 and WT rice seeds were analyzed by high-performance liquid chromatography, VB2 was detected according to the method of GB 5009.85-2016, and VE was quantified by GB 5009.82-2016. Fat was detected by GB 5009.6-2016 using a Soxhlet extractor. The ash content was measured by the gravimetric method after igniting the rice sample in a muffle furnace at a 600°C temperature for 12 h.

Minerals in rice seed were analyzed by atomic absorption spectroscopy (AAS) according to a previous study (Jiang *et al.* 2007). Approximately 2.0 g of brown seeds were obtained, placed in a crucible and ignited in a muffle furnace at 550–600°C for 10 h. The ash of the rice sample was dissolved in 0.2 N HCl and filtered using filter paper. The filtrate was used for AAS analysis with the respective hollow-cathode lamp.

2.9 *Statistical analysis*

All experiments were performed at least three times. Error bars in each graph indicated the mean values ± standard error of replicates. Statistically significant differences between measurements were determined through an independent sample *t*-test (*$P<0.05$; **$P<0.01$) by using IBM Statistical Product and Service Solutions (SPSS) Statistics software version 24 (IBM, USA).

3. Results

3.1 *Molecular analysis of the RSV-resistant transgenic plants*

Transgenic rice line with strong resistance against RSV was generated using a dsRNAi construct designed to silence the entire sequence region of RSV *SP*. The T_6 progeny of transgenic rice line SP-7 (renamed KRSV-1), which contained a p1300 expression vector, was used as the material for further analyses (Ma *et al.* 2011). Viral resistance assays showed that KRSV-1 conferred significantly enhanced RSV resistance (figure 1A and B).

Molecular analysis is one of the most important aspects to evaluate the biosafety of transgenic plants, which consists of a transgene copy number and T-DNA integration site detection. Southern blot analysis showed a single hybridization band in the KRSV-1 T_1 heterozygous plants in the present study (Ma *et al.* 2011). Southern blot hybridization was repeated with three types of single restriction digestions, which demonstrated that one copy of the transgene was present in KRSV-1 T_6 transgenic progeny (figure 1C). Stable inheritance can be obtained from single-

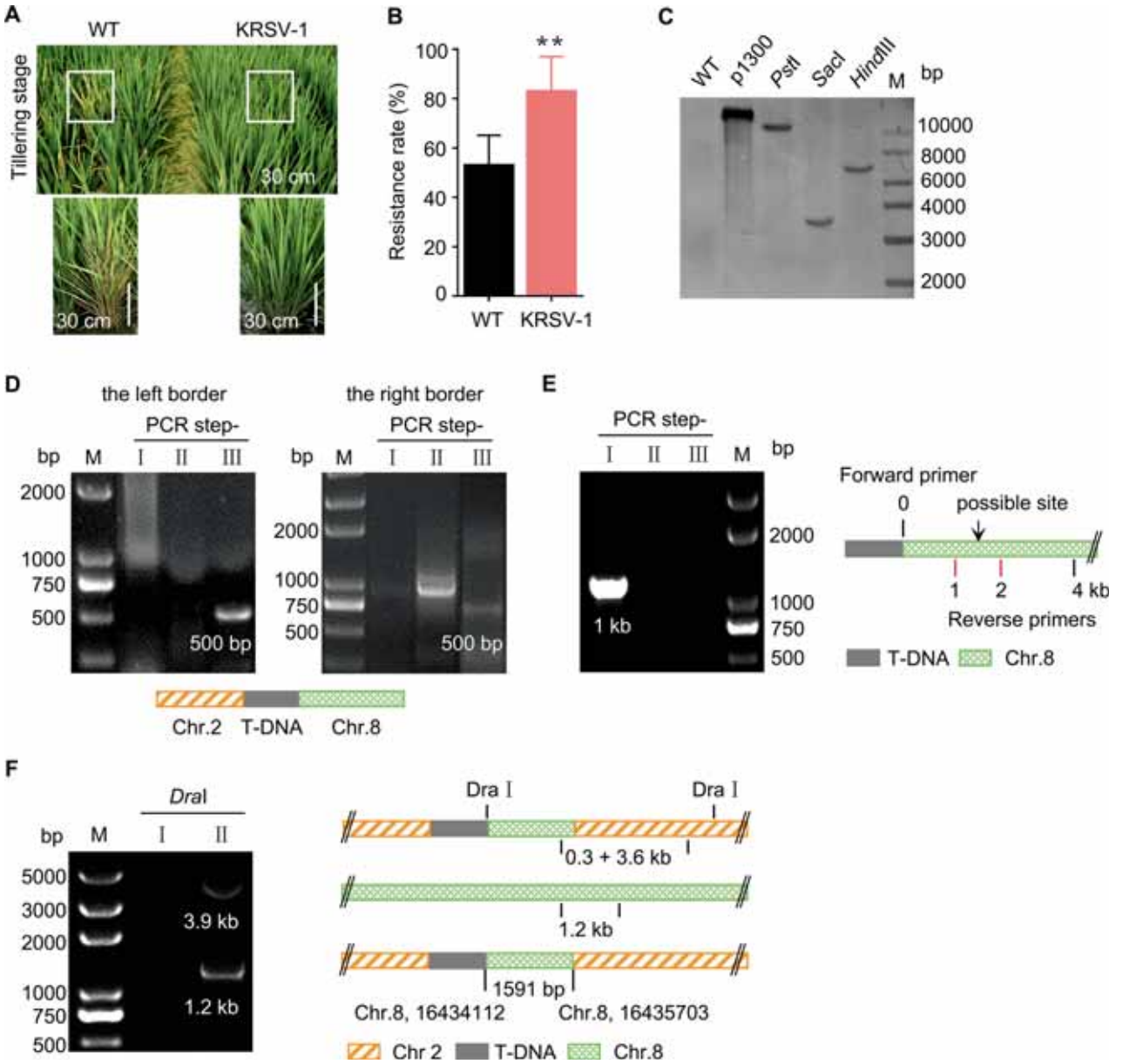

Figure 1. RSV resistance assays and molecular analysis of transgenic plants. (**A**) RSV-resistance assays of KRSV-1 and WT after viral infection. Plants were exposed to the viruliferous or virus-free insects of *L. striatellus* Fallen for 72 h. Then, the plants were grown in the experimental stations until the tillering stage (see section 2). The leaves of KRSV-1 were green, whereas mottled leaves were found in WT. Bottom: magnified image of transgenic plants and WT. Scale bars = 30 cm. (**B**) RSV resistance rate of self-fertilized progeny (T_6) of transgenic rice line after viral infection. Error bars indicate SEM. $^{**}P<0.01$ (Student's *t* test). These experiments were repeated three times, obtaining similar results (see section 2). (**C**) Transgene copy number analysis of transgenic plants with different enzyme digestions via Southern blot hybridization. Approximately 3.0 g of individual plants that had been transformed with p1300SP, p1300 or WT were used to extract DNA and then digested with different enzymes. WT, wild-type, from non-transgenic plants; p1300, p1300 plasmid; *Pst*I, *Sac*I and *Hind* III, genomic DNA extracted from KRSV-1 were digested with corresponding enzymes; M, marker. (**D**) The left and right borders of T-DNA obtained by hiTAIL-PCR. 500-bp amplified products were sequenced in Supplementary figure 1. The detailed PCR procedure was described in section 2. (**E**) Three primers 1-kb (I), 2-kb (II) and 4-kb (III) away from the right border of T-DNA were designed according to the sequence of chromosome 8 to analyze the right border of T-DNA. 1-kb amplified products were sequenced. Schematic illustrations of the T-DNA insertion site and used primers are exhibited on the right. Position of reverse primers used for amplifying the native chromosome 8 sequence was marked by the filled arrows, and the forward primer was designed based on the T-DNA sequence. (**F**) T-DNA carried 1591 bp segment of chromosome 8 (16,434,112–16,435,703) adjacent to the right border of T-DNA translocated into the intergenic region of 2,443,520–22,443,553 on a chromosome 2 non-coding region. Schematic illustrations of the T-DNA insertion site and the beginning and ending sites of flanking sequences in T-DNA right border are exhibited on the right. The left beginning site of T-DNA right border was obtained using figure 1D at 16,434,112. The right ending site of T-DNA right border was obtained by the 3.9-kb amplified PCR products at 16,435,703. RSV: *rice stripe virus*; hiTAIL-PCR: high-efficient thermal asymmetric interlaced PCR.

copy number of the transgene in transgenic plants, which provides their stable resistance (Wang *et al.* 2000; Ma *et al.* 2011). Combined with the strong RSV resistance of KRSV-1 as shown in figure 1A, these results showed that KRSV-1 is a relatively stable transgenic rice line, of which transgene and viral resistances can be stably inherited.

Analysis of the integration site of transgenic rice line by hiTAIL-PCR sequences revealed that the left border of T-DNA was flanked by a sequence on chromosome 2, while the right border was flanked by a sequence on chromosome 8 (nucleotide position 16,434,112–16,434,880) (figure 1D and supplementary figure 1). Genome-walking assays confirmed the results of the left border of T-DNA, suggesting that T-DNA was inserted into chromosome 2 (supplementary figure 2A and B). To further confirm the insertion site of foreign DNA fragments, we selected specific restriction enzymes that were both in the T-DNA and the adjacent rice genome to perform Southern blot analysis. The left-junction fragment was located on chromosome 2 as determined by restriction enzyme *Hind*III digestion (supplementary figure 2C). Moreover, *Dra*I and *Apa*I were selected to conduct the Southern blot analysis, and the results showed that the transgenic rice line was with inter-chromosomal rearrangement in the right border of T-DNA as compared with WT (supplementary figure 2D).

To further analyze the right border of T-DNA, three downstream primers which were 1-, 2- and 4 kb away from the right border of T-DNA were designed according to the sequence of chromosome 8. However, only one band was obtained using the primers of 1 kb in the PCR, which indicated that the breakpoint was located 1–2 kb away from the right border of the T-DNA on chromosome 8 (figure 1E). Genome walking and long-PCR were performed to determine the T-DNA breakpoint accurately, and a major amplified product of 3900 bp was yielded in the *Dra*I library, which contained the 300-bp region (nucleotide position 16,435,486–16,435,703) of chromosome 8 and the 3600-bp region of chromosome 2 (figure 1F and supplementary figure 3B). All the results showed that T-DNA carried the 1591-bp segment of chromosome 8 (nucleotide position 16,434,112–16,435,703) adjacent to the right border of the T-DNA translocated into the intergenic non-coding region of chromosome 2 at the nucleotide position 2,443,520–22,443,553 (figure 1F and supplementary figure 3B).

However, the shorter sequence only contained the native 1.2-kb sequence between positions 16,435,486 and 16,436,733 of chromosome 8, suggesting that the translocated fragments remained intact at the native locations on chromosome 8 (figure 1F and supplementary figure 3A). Accordingly, due to the transformation, the 32-bp fragments of host-genome sequences were replaced by T-DNA insertions, and a 3-bp filler sequence GCA was present at the junction of chromosomes 2 and 8 (supplementary figure 3). These results suggested a possible inter-chromosomal rearrangement in which the T-DNA carried a 1591-bp region of chromosome 8 and translocated into the intergenic region of chromosome 2.

3.2 *Unintended effects are not detected in KRSV-1 plants by transcriptomics analysis*

To investigate the transcriptional responses or unintended effects of transgenic plants due to transgene insertion, transcriptomics analysis was performed using the RNA from transgenic rice line and WT plants. The method efficiently analyzed the data, because the plateau was quickly reached after 2,000,000 tags were sequenced (figure 2A). After filtering the adaptor sequences and removing low-quality tags, approximately 28,774 unique tags were aligned to the rice genome and were used for further analysis (supplementary data 1). The digital gene expression tag profiling was determined by comparing the genes expressed in KRSV-1 and WT at $P \leq 0.05$ and an expression change of 2-fold or more ($|\log2\text{Foldchange}| \geq 1$). We obtained 7434 DEGs, where 4436 transcripts were induced, and 2998 were repressed (figure 2B, marked in yellow in supplementary data 1). Only 650 DDEGs with expression differences above 5-fold were identified, approximately 6.4% (473) of the total unique tags dramatically increased, and 2.4% (177) decreased in the libraries (figure 2B, marked in red in supplementary data 1). The expression levels of the rest of the unique tags (6783) were within 5-fold differences (figure 2B and supplementary data 1).

Based on the classification standard of the GO analysis, the DEGs of KRSV-1 *vs* WT plants were classified into three major categories associated with biological process, cellular components and molecular function (figure 2C–E). In the cellular component term of the GO analysis, most DEGs were found in the subcategory plastid (figure 2D). The chloroplast is an organelle specialized where photosynthesis takes place in plants and eukaryotic algae. Many DEGs in the chloroplast might be related to basic fundamental photosynthetic acclimation of transgenic plants. From the biological process analysis, apart from the metabolic and biological terms responsible for basic cellular activities, most of the other DEGs respond to stress, including the dicer-like gene or other exoribonuclease that functions in RNA-mediated viral resistance, downstream LRR family genes and pathogenesis-related proteins function in defense response (figure 2C and supplementary data 1). Chromosome localization analysis exhibited that the differentially expressed mRNAs in transgenic rice were distributed to all 12 chromosomes and did not focus on the inserted portion on chromosome 2 (figure 2F). Overall, no detrimental changes were observed in the transgenic plants based on transcriptomics analysis.

3.3 *Confirmation of transcriptomics data by qRT-PCR*

To further verify the transcriptomics data, nine dramatically differentially expressed members were selected for qRT-PCR analysis. *Dicer 3-like protein* is an important component of RNAi pathway (Wilson and Doudna 2013). Heat-shock

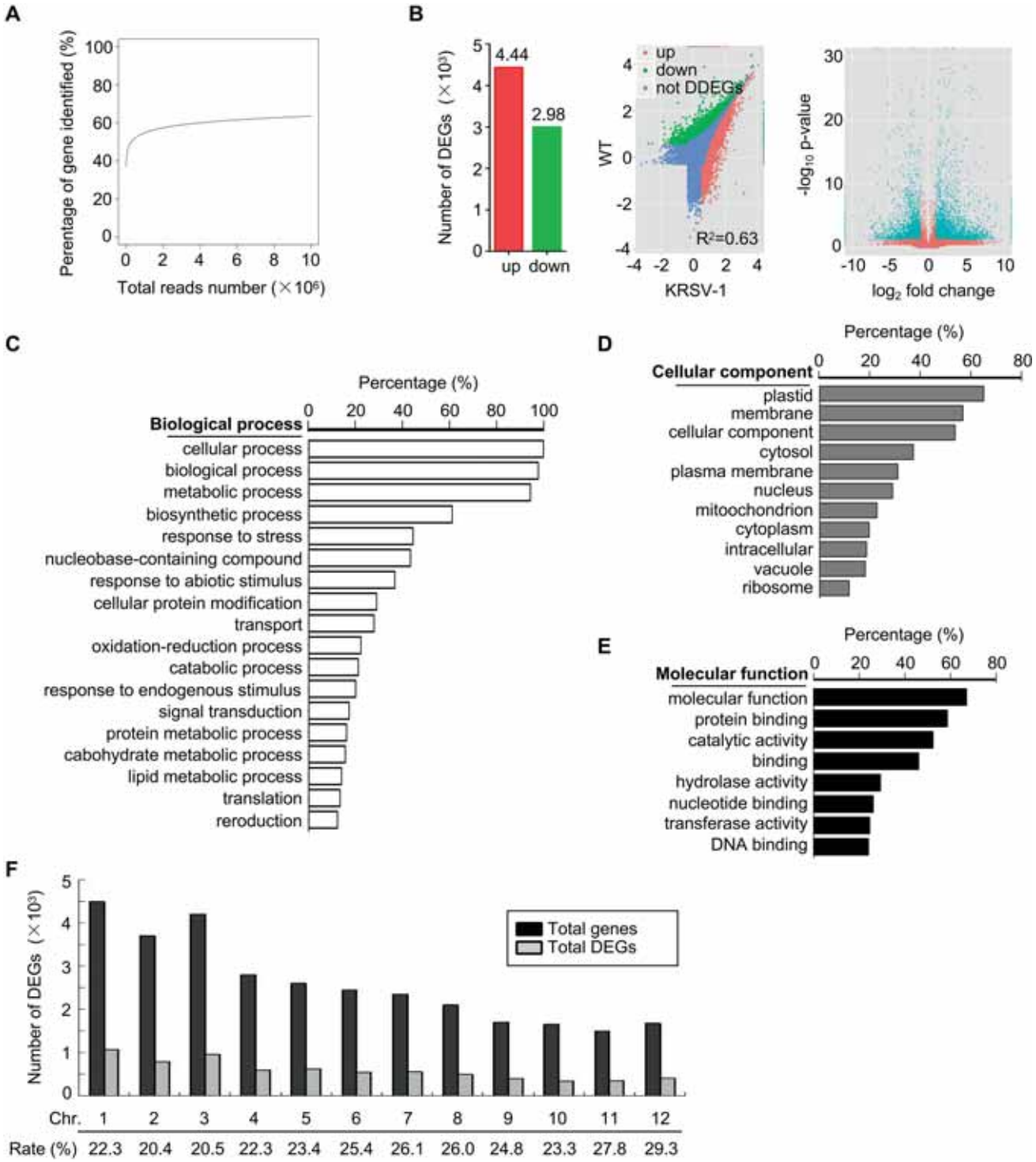

Figure 2. Transcriptomics analysis of the transgenic and WT plants. (**A**) Accumulation of the genes mapped by unique clean tags in our libraries. Percentage of genes identified (*y*-axis) increases as the total tag number (*x*-axis) increases. (**B**) Comparison of DEGs between the two libraries. Image on the left represents the numbers of DEGs. Blue dots represent the transcripts with no significant expression in the middle image, and red and green dots represent upregulated and downregulated DDEGs. Cufflink dots in the right image were related to the DEGs with differential expression. (**C**) Biological process GO terms of DEGs in KRSV-1 and WT plants via transcriptomics analysis. Numbers indicate the proportion of DEGs in each category. (**D**) Cellular component GO terms of DEGs in KRSV-1 and WT plants via transcriptomics analysis. Numbers indicate the proportion of DEGs in each category. (**E**) Molecular function GO terms of DEGs in KRSV-1 and WT plants via transcriptomics analysis. Numbers indicate the proportion of DEGs in each category. (**F**) Distribution of DEGs on different chromosomes. Total genes, the total number of genes on the chromosome. Total DEGs, identified DEGs in different chromosomes. DEGs: differentially expressed genes; DDEGs: dramatically differentially expressed genes; GO: gene ontology.

protein, chaperonin and pathogenesis-related protein as protective molecules are involved in stress response (Sarowar *et al.* 2005; Li *et al.* 2011). The other statistically significantly differential members function in basic cellular mechanisms and signaling transduction. The results showed that except for the repressed gene, *NADH dehydrogenase subunit 9*, the other members accumulated in KRSV-1 (figure 3A–I), which might be associated with the potential RNAi-mediated defense response in RSV-resistant transgenic plants. The results indicated that all gene expression patterns from qRT-PCR were consistent with those from the transcriptomics analysis.

3.4 *No unintended effects are detected by proteomics analysis*

Rice seeds, as the main edible portions, bear the brunt of safety assessment in GM plants. We thus performed a whole proteomics analysis of the stable transgenic rice line KRSV-1 rice seeds. No significantly different bands were observed in the protein samples from KRSV-1 and WT under SDS-PAGE experiment (figure 4A). Supplementary data 2 shows that 1888 proteins were detected, and 357 DEPs showing fold changes ($P<0.05$) were considered in the following study. Then, DEPs were classified into three major GO categories associated with molecular function, biological process and cellular component (figure 4B–D). Most DEPs in the proteomics data were involved in basic cellular metabolic processes, which was consistent with the DEGs in the transcriptomics data.

To further determine the functional role of DEPs responsible for RSV resistance and unintended effects, Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis was performed. The nucleic acid binding and hydrolase class of proteins were most abundant, which functioned in basic metabolism, including replication, transcription and protein-processing in the protein class term (figure 4E). Moreover, most DEPs participated in cytoskeletal regulation and different signaling pathway to maintain normal life from the pathway ontology analysis (figure 4F). The differentially expressed members in the transcriptomics and proteomics analyses had a strong correlation with the basic cellular processes. No altered members were toxic, allergenic or detrimental to the growth capabilities of the transgenic rice.

3.5 *No detrimental toxic protein and allergens are observed in the KRSV-1 transgenic rice*

Food allergy, as an important health issue, has been given much concern. In rice, proteins with molecular masses of

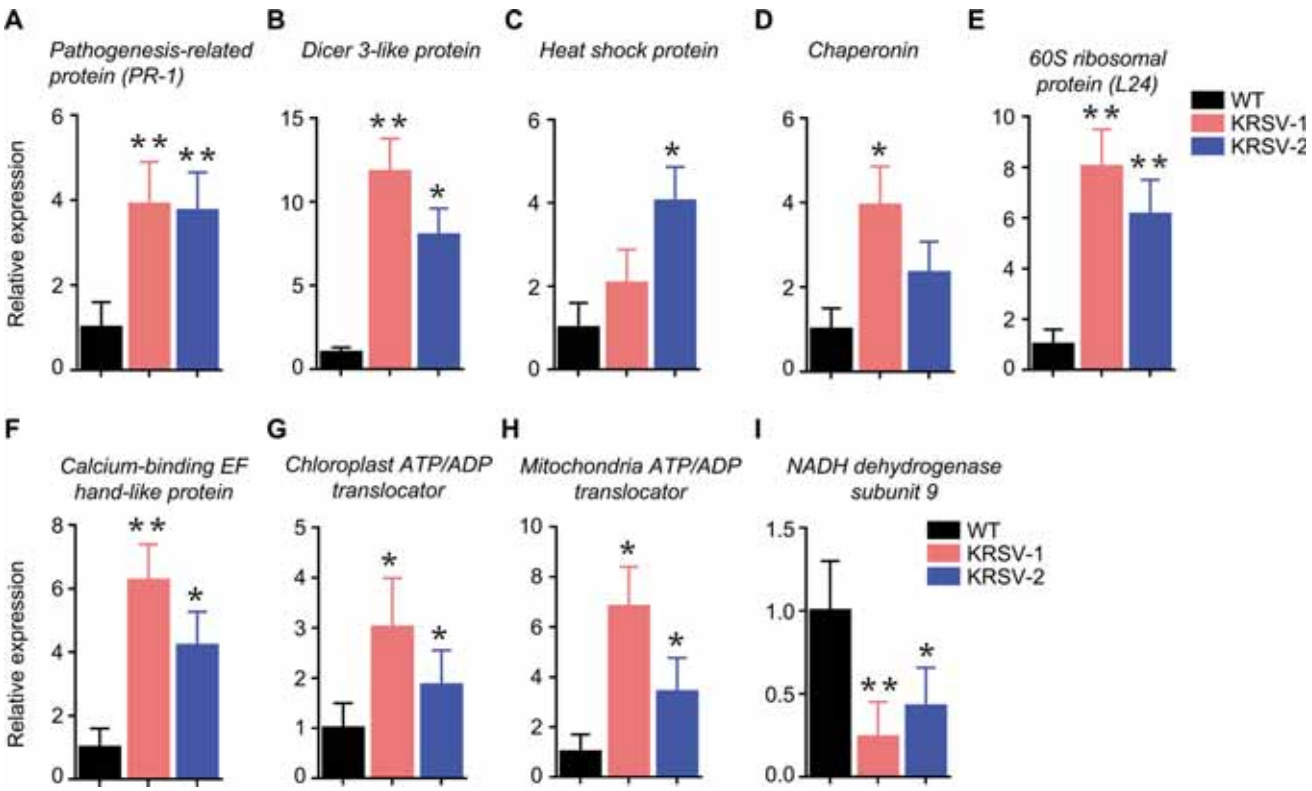

Figure 3. qRT-PCR analysis of DDEGs. (**A–I**) Expression patterns of nine DDEGs in transcriptome were confirmed by qRT-PCR in the transgenic rice line and WT plants. Relative abundance of nine DDEGs was normalized against *Osactin*. Error bars indicate SEM. *$P<0.05$; **$P<0.01$ (Student's *t* test). Statistical analysis was based on three independent biological repeats. qRT-PCR: quantitative real-time PCR; DDEGs: dramatically differentially expressed genes.

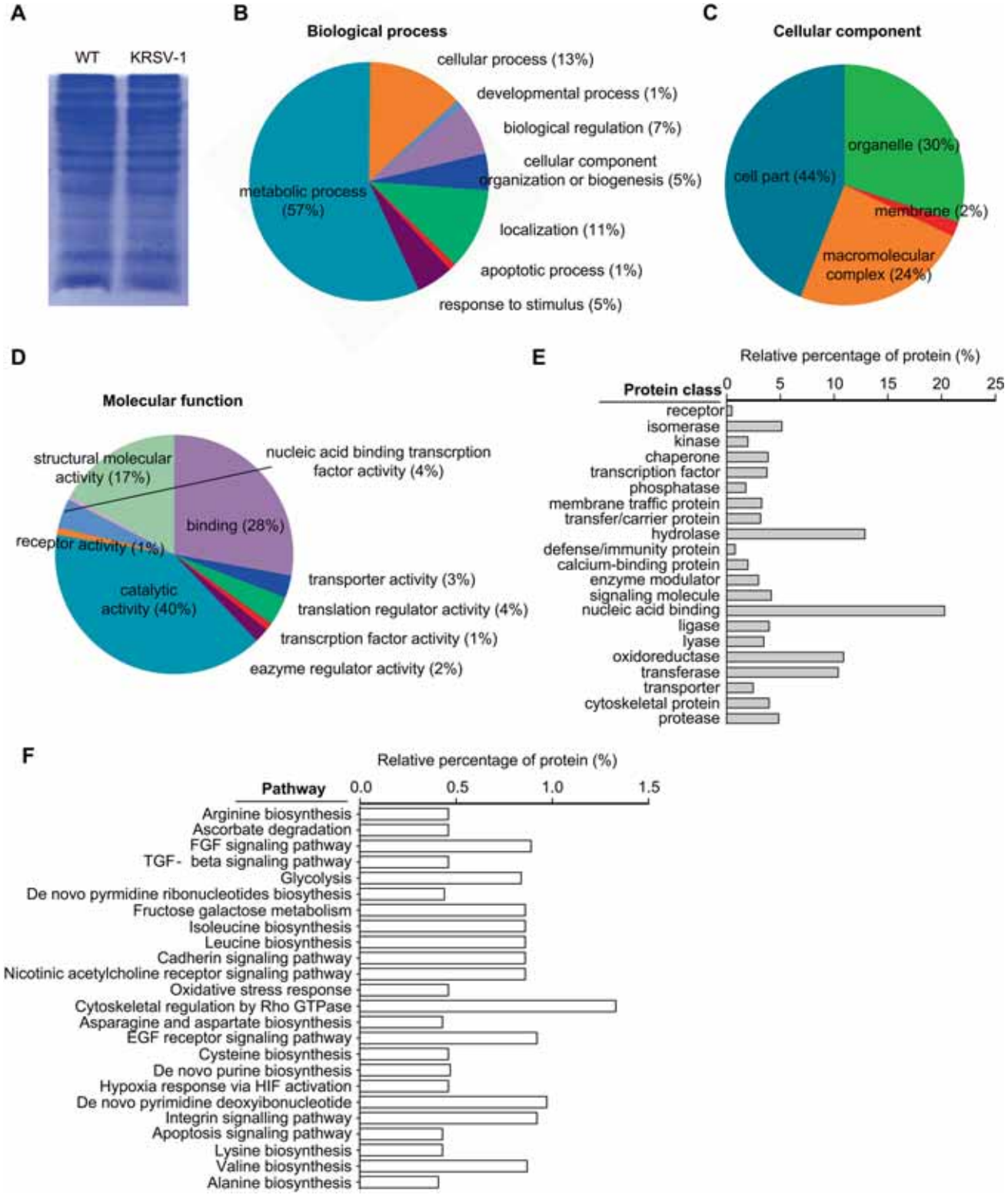

Figure 4. Proteomics analysis of the transgenic line and WT plants. (**A**) SDS-PAGE analysis of the protein samples from KRSV-1 and WT seeds. (**B–D**) Biological process, cellular component and molecular function GO terms of DEPs and ratios in KRSV-1 and WT plants. Numbers indicate the proportion of DEPs in each category. (**E**) Protein classes of DEPs based on KEGG analysis. Numbers indicate the proportion of DEPs in each category. (**F**) Pathways of DEPs based on KEGG analysis. Numbers indicate the proportion of DEPs in each category. DEPs: differentially expressed proteins; GO: gene ontology; KEGG: Kyoto Encyclopedia of Genes and Genomes.

14–16, 26, 33 and 56 kDa are potentially allergenic (Usui *et al.* 2001). Most of the known and candidate rice allergens were selected to blast in the transcriptomics and proteomics data (table 1). Apart from two allergens (Glb33 LOC_Os08g09250.2 and Bet v 1-D/H LOC_Os12g36860), other well-known allergens were not observed in 28,774 mRNAs by transcriptomics analysis and 1888 proteins by proteomics analysis. LOC_Os08g09250.2 as a 33-kDa allergen, designated Glb33, had a strong IgE binding ability to cause IgE-mediated atopic diseases, such as allergic rhinitis and seasonal asthma (Usui *et al.* 2001). No statistically significant differences in LOC_Os08g09250.2 ($P = 0.37198 > 0.05$) were observed between the rice samples, which was only slightly higher in the transgenic seeds within the appropriate range (table 1). Another member Bet v 1-D/H was a significantly different allergen ($P = 0.01098 < 0.05$), which belong to the Bet v 1 family, having a strong IgE binding ability (Ogo *et al.* 2014). Bet v 1 was dramatically lower in KRSV-1 than in WT (table 1). Detrimental toxic protein and allergens were not observed in the KRSV-1 transgenic rice through transcriptomics and proteomics analyses.

3.6 *Transgenic rice line KRSV-1 shows proximate compositions with WT*

We conducted a comprehensive biosafety evaluation of various components including amino acids, protein contents, vitamins, ash, moisture, crude fat, crude fiber, inorganic ions and heavy metal ions of transgenic rice line. The protein contents of KRSV-1 (9.04%) were slightly higher than those of WT (7.76%), whereas crude fat was relative lower in KRSV-1 (1.37%) than in WT (1.84%). The amino acid contents of KRSV-1 were almost indistinguishable from those of non-transgenic rice seeds (table 2).

Inductively coupled plasma mass spectrometry afforded a precise handle on the contents of inorganic ions. Only small variations in Na, K, Ca and Mg compositions were observed. The quantification of heavy metals (Hg, Cd, Pb and Mn) in transgenic rice seeds was also almost indistinguishable from that of non-transgenic plants. Aside from the nutrients above, vitamins (apart from VB1), ash, moisture and crude fiber were also checked, and no statistically significant difference was discovered (table 2). Thus, all the data demonstrate that the transgenic rice line is nutritionally competitive with WT.

4. Discussion

The development of transgenic crops offers immense opportunities to improve crop products, disease or abiotic stress resistance and nutrient quality by incorporating genes of interest into crops (Clive 2007). However, potential safety issues resulting from the genetic modification of transgene are also among the key issues in the national economy and people's livelihoods. Genetic products should be subjected to detailed biosafety assessment before entering the market (Conner and Jacobs 1999; Ren *et al.* 2009). Biosafety assessment of GM plants includes evaluating their molecular characteristics and unintended effects in a structured, stepwise and comparative approach (Conner and Jacobs 2000).

The integration pattern and copy number of T-DNA fragments are two broad aspects of molecular analysis, which affect not only the transformation efficiency and stability but also the expression properties of the transgenes (Wang *et al.* 2000). The T-DNA-tagged rice in our study harbored a single copy of the T-DNA as verified by Southern blot analysis, and self-fertilized homozygous T_6 progeny transgenic rice line displayed RSV resistance, suggesting that the transgene was stably inherited in the transgenic rice line (figure 1). *Agrobacterium* T-DNA integration in plants is a very complex process, which has not been fully understood. T-DNA integration in KRSV-1 in our study carried the partial fragments of chromosome 8 to translocate into chromosome 2, which caused inter-chromosomal rearrangements (figure 1). This phenomenon might occur when the right and left borders of one T-DNA were integrated into chromosomes 2 and 8 simultaneously, which was very close in space in the nucleus when the T-DNA was inserted. This phenomenon could result in the formation of a transient T-DNA bridge between the two chromosomes. Subsequently, the aberrant T-DNA structure was excised from that site with portions of chromosome 8 at the right border and then integrated into chromosome 2. Moreover, inter-chromosomal translocations caused by T-DNA integration might generally be accompanied by duplications as described in the inter-chromosomal translocation of T-DNA in the previous study (Tax and Vernon 2001).

T-DNA inserts randomly within the genome, but it has some bias toward inserting into transcribed regions (Tax and Vernon 2001), which may lead to physical damage of the plant genome, such as the inactivation of endogenous genes, the disruption of *cis*-regulating elements, or the modification of biochemical pathways (Ren *et al.* 2009). Analysis of the junctions between the T-DNA ends by hiTAIL-PCR and genome walking showed that the T-DNA carried partial fragments of chromosome 8 into the intergenic region of chromosome 2 (figure 1). Moreover, the DEGs in transgenic rice did not localize into the insertion site of chromosome 2 and were almost equally distributed on all 12 chromosomes by transcriptomics analysis (figure 2F). The insertion of the T-DNA into non-coding regions might not disrupt the preexisting expression characteristics of transgenic plants.

The assessment of unintended effects, which emerges due to the secondary or pleiotropic effects of transgene expression and insertional effects, is the most important and arduous parts of biosafety evaluation (Kuiper *et al.* 2001). Considering that the public mainly focuses on the unintended effects of GM plants that affect people, non-targeted technologies such as analytical approaches at the transcript,

Table 1. Blast analysis of known candidate allergens in transcriptomics and proteomics data

Annotation/locus ID	NCBI No.gi	Reference	RNA-seq WT	RNA-seq KRSV-1	Proteome WT	Proteome KRSV-1	Potential risk
LOC_Os07g11370	1002280824	Alvarez *et al.* (1995)	–	–	–	–	No
LOC_Os08g09250.2	16580747	Usui *et al.* (2001)	1	1.095^{ns}	–	–	No
RAG1 LOC_Os07g11360	218198	Adachi et al. (1993)	–	–	–	–	No
LOC_Os07g0215500	1398917	Alvarez *et al.* (1995)	–	–	–	–	No
RA17	218194	Adachi et al. (1993)	–	–	–	–	No
RA14	218192	Adachi et al. (1993)	–	–	–	–	No
LOC_Os12g36860	1002314013	Ogo *et al.* (2014)	1	0.11284 $p = 0.01$	–	–	No
LOC_Os12g36850	1002310882	Ogo *et al.* (2014)	–	–	–	–	No
Ory s1 LOC_Os03g01610	8118420	Xu *et al.* (1995)	–	–	–	–	No
Ory s12	1087919564	Russell *et al.* (2018)	–	–	–	–	No
LOC_Os03g0793700	115455865	Satoh *et al.* (2011)	–	–	–	–	No
Putative globulin	41469581	Satoh *et al.* (2011)	–	–	–	–	No
Globulin-like protein	34495244	Satoh *et al.* (2011)	–	–	–	–	No
19 kDa globulin precursor	20159	Satoh *et al.* (2011)	–	–	–	–	No
Allergenic protein	218197	Satoh *et al.* (2011)	–	–	–	–	No
LOC_Os07g0216700	115471187	Satoh *et al.* (2011)	–	–	–	–	No
LOC_Os08G0560700	1002280849	Hirano *et al.* (2013)	–	–	–	–	No
LOC_Os06G0556600	1002276622	Hirano *et al.* (2013)	–	–	–	–	No
LOC_Os10G0323900	1002300730	Hirano *et al.* (2013)	–	–	–	–	No
LOC_Os06G0545400	1002275617	Hirano *et al.* (2013)	–	–	–	–	No

Transcriptome: 28,774 mRNAs; Proteome: 1888 proteins; –: not detected in database; ns: no significance

Table 2. Various component analysis of KRSV-1 and WT rice seeds

		WT	KRSV-1	Statistical analysis
Amino acids (% fresh weight)	Asp (D)	0.7 ± 0.1	0.7 ± 0.2	ns
	Thr (T)	0.2 ± 0.1	0.2 ± 0.1	ns
	Ser (S)	0.3 ± 0.1	0.3 ± 0.1	ns
	Glu (E)	1.3 ± 0.2	1.3 ± 0.2	ns
	Gly (G)	0.4 ± 0.1	0.4 ± 0.1	ns
	Ala (A)	0.4 ± 0.1	0.5 ± 0.1	ns
	Cys (C)	0.1 ± 0.1	0.1 ± 0.1	ns
	Val (V)	0.5 ± 0.1	0.6 ± 0.1	ns
	Met (M)	0.2 ± 0.1	0.3 ± 0.1	ns
	Ile (I)	0.3 ± 0.1	0.4 ± 0.1	ns
	Leu (L)	0.6 ± 0.2	0.7 ± 0.2	ns
	Tyr (Y)	0.3 ± 0.1	0.3 ± 0.1	ns
	Phe (F)	0.4 ± 0.1	0.4 ± 0.1	ns
	His (H)	0.2 ± 0.1	0.2 ± 0.1	ns
	Lys (K)	0.3 ± 0.1	0.3 ± 0.1	ns
	Arg (R)	0.6 ± 0.2	0.6 ± 0.1	ns
Total protein (%)	Protein	7.76 ± 0.71	9.04 ± 0.83	*
Inorganic ion (mg/kg) (fresh weight)	Plumbum	0.18 ± 0.02	0.18 ± 0.02	ns
	Calcium	445.62 ± 42	477.11 ± 46.3	ns
	Potassium	3202.3 ± 360	3626.5 ± 400	ns
	Sodium	15.88 ± 2.1	17.92 ± 1.9	ns
	Magnesium	987.1 ± 99	1087.8 ± 92	ns
Heavy metal ion (mg/kg) (fresh weight)	Cadmium	0.009 ± 0.001	0.009 ± 0.001	ns
	Mercury	<0.005 ± 0.001	<0.005 ± 0.001	ns
	Zinc	19.48 ± 2.0	19.12 ± 1.8	ns
	Copper	4.16 ± 0.4	4.15 ± 0.3	ns
	Manganese	4.99 ± 0.5	5.13 ± 0.46	ns
	Iron	0.74 ± 0.07	0.65 ± 0.06	ns
Vitamin (mg/kg) (fresh weight)	VE	0.862 ± 0.081	0.853 ± 0.072	ns
	VB1	0.191 ± 0.020	0.258 ± 0.021	*
	VB2	0.013 ± 0.001	0.013 ± 0.001	ns
Other components (%)	Crude fat	1.84 ± 0.2	1.37 ± 0.15	*
	Moisture	8.95 ± 1.0	8.84 ± 0.8	ns
	Crude fiber	7.19 ± 0.8	7.91 ± 0.9	ns
	Ash	3.86 ± 0.3	3.56 ± 0.4	ns

Statistics based on three independent biological samples (Student's *t* test, *$P<0.05$). ns: no significance

protein and metabolite levels are the methods-of-choice for the comprehensive evaluation of GM plants' biosafety (Rischer and Oksman-Caldentey 2006). In this study, transcriptomics and proteomics approaches were adopted to compare the differences in transgenic rice with WT. Although the results revealed some levels of alteration in the mRNA and protein profiles of the KRSV-1, none of the altered members were toxic, allergenic or detrimental substances to the growth capabilities of the transgenic rice (table 1 and figures 2–4).

Differentially expressed members from the transcriptome and proteome may be caused by various reasons. The insertion and integration of a transgene can induce insertion, position and transformation effects (Filipecki and Malepszy 2006). In addition, the expression of transgenes in transgenic plants also causes pre-existing sequence changes. Based on the comprehensive analysis of transcriptomics and proteomics data, basic metabolisms including photosynthesis, replication or transcription and oxidative respiration or energy transfer were activated more in transgenic plants than that in WT, which might compensate for transgene-associated changes in metabolic loads (figures 2–4). Similar results were obtained in other transgenic plants in previous study (Ren *et al.* 2009). Additionally, the data analysis revealed that the majority of transgenic plants were differentially expressed, and upregulated members of the KRSV-1 transgenic rice were involved in the RNAi silencing pathway and downstream plant defense mechanisms, thus contributing some possible advantages to these transgenic plants (figures 2–4). Our study was in accordance with the recent findings that DEPs in the transgenic plants might not be identified as new proteins or allergens but rather as resistance-changed proteins (Ruebelt *et al.* 2006; Mishra *et al.* 2017).

The genetic transformation by *Agrobacterium tumefaciens*-mediated T-DNA integration was applied to generate transgenic plants (Yang *et al.* 2011). In our study, different techniques including the functional genome, transcriptome, proteome and nutritional component profile were employed to characterize the changes resulting upon the insertion of

T-DNA in the genome. Unintended effects in the form of new toxins or allergens were not detected in transgenic plants by using the current techniques, and the nutritional components were competitive with WT (tables 1 and 2). We cannot rule out the possibility that a more sensitive approach such as metabolomics analysis will reveal subtle unintended changes from the insertion of the transgene.

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