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Characteristics of Metabolites by Seed-Specific Inhibition of *FAD2* in *Brassica napus L.*

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Running title: Seed-Specific down-expression of *FAD2* is suitable to obtain high oleic acid from *B. napus.*

ABSTRACT: Fatty acid desaturase-2 (FAD2) is a key enzyme in the production of polyunsaturated fatty acids in plants. RNAi technology can reduce the expression of *FAD2* genes in *B. napus* seeds and acquire transgenic *B. napus* plants with a high oleic acid content, but the effect of seed-specific inhibition of *FAD2* expression on *B. napus* seed metabolites is not clear. Here we use widely targeted metabolomics to investigate the metabolites of normal oleic-acid rapeseed (OA) and high oleic-acid rapeseed (HOA) seeds, resulting in a total of 726 metabolites being detected. Amongst them, 24 differential metabolites were significantly down-regulated, and 88 differential metabolites were significantly up-regulated in HOA rapeseed. The differential metabolites were annotated by the KEGG database. Most of these differential metabolites were annotated in metabolic pathways and biosynthesis of secondary metabolites; purine, phenylalanine and glyceride were significantly revealed by the KEGG enrichment analysis. At the same time, glycolipids and phospholipids in *B. napus* seeds were also accurately quantified. The lipid content of C18:1 increased significantly whilst that of C18:2 significantly decreased in HOA rapeseed because *FAD2* expression was inhibited. The original balance of lipid biosynthesis was completely changed, which in turn altered gene expression related to the Kennedy pathway. The insertion site (chromosome A09, non-coding region) of the intron-spliced hairpin RNA (ihpRNA) plant expression vector was reconfirmed through genome-wide resequencing, and the transgenic event did not change the sequence of *FAD2* genes. There was no significant difference in germination rate and germination potential between OA and HOA rapeseed seeds because the seed-specific ihpRNA plant

expression vector did not affect other stages of plant growth. This work provides a theoretical and practical guidance for subsequent molecular breeding of HOA *B. napus*.

KEYWORDS: *Brassica napus L.*, RNAi, Metabolomics, Fatty Acid Desaturase-2

INTRODUCTION

Brassica napus L.(Rapeseed) belongs to the Brassicaceae family, and it is one of the most important oil crops in the world[1]. China is one of the largest producing countries, with rapeseed oil being the largest source of vegetable oil[2]. Rapeseed oil contains high levels of polyunsaturated fatty acids, such as linoleic acid (18:2) and linolenic acid (18:3)[3]. The chemical properties of these fatty acids are very unstable and are easily oxidized to form substances harmful to human health. Nevertheless, oleic acid (18:1) is relatively stable, having high thermal stability and therefore has the important function of reducing cardiovascular disease[4]. Consequently, an important goal for plant breeders is to increase the content of oleate in rapeseed oil, whilst at the same time, reducing the content of polyunsaturated fatty acids[5-9].

The synthesis and metabolism of fatty acids in plants is a complex process, which mainly includes the extension of carbon chains and the desaturation of chemical bonds. Fatty acid desaturase 2 (FAD2) on the endoplasmic reticulum is a key enzyme in the production of polyunsaturated fatty acids in plants. It introduces a second double bond to oleic acid (18:1) binding to phosphatidic acid choline to form linoleic acid, which is the first step in the synthesis of polyunsaturated fatty acids[10, 11]. At the same time, dihydroxyacetone phosphate, the intermediate product of glycolysis, is catalyzed by glycerol 3-phosphate dehydrogenase (GPD) to form glycerol 3-phosphate, the skeleton component of triglyceride (TAGs). Then, through the Kennedy pathway, acyl coenzyme A synthesizes triacylglycerol via the catalysis of 3-phosphoglyceryl acyltransferase (GPAT), hemolytic phosphatidic acid acyltransferase (LPAT),

phosphatidic acid phosphohydrolase (PAP) and diacylglycerol transferase (DGAT) on the endoplasmic reticulum[12, 13].

In this study, the intron-spliced hairpin RNA (ihpRNA) plant expression vector that contains the seed-specific promoter napinA and the reverse repeat box of *FAD2* gene fragment, was selected and transferred into wild-type *B. napus*. In the genetic transformation mediated by *Agrobacterium tumefaciens*, the integration of foreign genes into the plant genome through T-DNA is random, so it is necessary to locate the insertion sites and analyze the effects on the plant genome. The next step was to study the effects of RNAi on plant metabolites.

Genome-wide resequencing is the DNA sequencing of species with known genomic sequences, based on which individual or population analysis is completed[17-19]. A large amount of variation can be detected by genome-wide resequencing, including single nucleotide polymorphism (SNP), insertion deletion (InDel), structural variation (SV) and copy number variation (CNV). In this study we re-sequenced the whole genome of wild-type *B. napus* and several generations of high OA RNAi transgenic *B. napus*, based on the detected variation that can help to find the insertion site more accurately.

The ihpRNA expression vector with *FAD2* genes as the target gene and seed expression specificity can inhibit the expression of *FAD2* genes in *B. napus* [20]. However, the effect of reducing *FAD2* gene expression on plant seed metabolites is largely unknown. Traditionally, high performance liquid chromatography (HPLC) and MS, or even UPLC-Q-TOF-MS are used to analyze various metabolites in plants[21, 22]. However,

these methods can only detect a small number of metabolites and cannot systematically and comprehensively describe the metabolites of plants or seeds. In recent years, metabolomics based on UPLC-ESI-MS/MS has attracted wide attention in the field for identification of plant metabolites because of its high throughput, rapid separation, high sensitivity and wide coverage. To date, this method has been widely used in the analysis of metabolites in corn, rice, tomato, potato and other plants[23-27], being an effective method to comprehensively understand plant secondary metabolites. Here we have studied glycolipids, phospholipids and widely targeted metabolites of wild-type *B. napus* and several generations of high oleic acid (HOA) RNAi transgenic *B. napus*. Analysis of the differences between these metabolites will be helpful for future *B. napus* breeding investigations.

MATERIALS AND METHODS

Plant Materials. Two *B. napus* genotypes, one having high oleic acid content (HOA) and Westar, wild-type having normal oleic acid content (OA) were used in the present study; *FAD2* in HOA was silenced and therefore showed higher OA content. The two genotypes were provided by Jiangsu Academy of Agricultural Sciences. HOA has been bred for many generations, and mature seeds of different generations were used in the following experiments. Storage time for each generation of seeds collected each year was the same. Approximately 100 pest-free mature seeds were surface sterilized with 10% bleach containing 0.1% Tween 20 for 15min. This was followed by 3 separate washings with sterilized water. The seeds were then sown on water-saturated

paper on plates, which were kept in the dark at 4°C for 3 d to improve germination uniformity before being transferred to a growth chamber with a 16-h-light (22°C)/8-h-dark (21°C) cycle. Germination took a total of 7 days, with each germination experiment being repeated three times: germination potential (%) = number of seeds germinated on day 5 / number of seeds tested × 100%, germination rate (%) = number of seeds germinated on day 7 / number of seeds tested × 100%[28]. After the seedlings had established, fresh leaves at the bolting stage were taken for the whole genome resequencing experiment.

Extraction of Total RNA and Real-time qPCR. To extract total RNA, a RNAprep Pure plant total RNA extraction kit (TIANGEN) was used. The integrity and concentration of RNA were detected by agarose gel electrophoresis and a microspectrophotometer. The first strand cDNA was synthesized from 2 μg total RNA by reverse transcription using an RT6 cDNA synthesis kit (TsingKe) with gDNA remover. The qPCR reaction was carried out in 384 well blocks by the SYBR green method using a Roche LightCycler Real-time 480 PCR system. The cycle threshold (Ct) value of each sample was treated with $2^{-\Delta\Delta Ct}$ [29]. All RT-qPCR reactions were carried out using total RNA samples separated from three independent repetitive samples with similar results. Specific primers of selected genes and *Actin*7 are listed in Table S1.

DNA extraction, library construction and sequencing. Plant genomic DNA was extracted by the CTAB method and agarose gel electrophoresis was used to analyze the degree of DNA degradation and whether there was RNA and protein contamination. Qubit was used to accurately quantify the concentration of DNA, in which the DNA

140 samples with contents above 0.5 μg was used to build the database. The genomic DNA
141 was randomly broken into fragments of about 350 bp by a Covaris fragmentation
142 instrument. After terminal repair and addition of a tail, the DNA libraries were
143 connected at both ends of the fragments. After the construction of the library, Qubit 2.0
144 was used for preliminary quantification, and then an Agilent 2100 was used to detect
145 the insert size of the library. After the insert size met the expectations, the qPCR method
146 was used to accurately quantify the effective concentration (3 nM) of the library and to
147 ensure its quality. The library was qualified, and the Illumina platform was used for
148 sequencing according to the effective concentration of the library and the data output
149 demand.

150 The original image data generated by the sequencing machine were converted into
151 sequence data via base calling (Illumina pipeline CASAVA v1.8.2) and then subjected
152 to quality control (QC) procedures to remove unusable reads:1) the reads contained the
153 Illumina library construction adapters, 2), the reads contained more than 10% unknown
154 bases (N bases) and 3), one end of the read contained more than 50% of low-quality
155 bases (sequencing quality value≤5). Sequencing reads were aligned to the reference
156 genome using BWA with default parameters. Subsequent processing, including
157 duplicate removal was preformed using samtools and PICARD
158 (http://picard.sourceforge.net). The raw SNP/InDel sets were named by samtools with
159 the parameters as "-q 1 -C 50 -m 2 -F 0.002 -d 1000". This set was then filtered using
160 the following criteria: (1) the mapping quality >20, and (2), the depth of the variate
161 position >4. Breakdancer and CNVnator were used for SV and CNV detections,

respectively. ANNOVAR was used for functional annotation of variants. The UCSC known genes were used for gene and region annotations[30-35].

Extraction and determination of glycolipids and phospholipids. Total lipid extraction was performed as described previously[36]. The content of lipids was determined by electrospray ionization tandem mass spectrometry (ESI-MS/MS)[37]. The data obtained in this study were from the SD averages of four independent samples, each of which was collected from multiple *B. napus* seeds.

Metabolic profiling. Metabolite profiling was carried out using a widely targeted metabolome method by Wuhan Metware Biotechnology Co., Ltd. (Wuhan, China) (http://www.metware.cn/). The freeze-dried seed was crushed using a mixer mill (MM 400, Retsch) with a zirconia bead for 1.5 min at 30 Hz. 100mg powder was weighed and extracted overnight at 4°C with 0.6 ml 70% aqueous methanol. Following centrifugation at 10,000g for 10 min, the extracts were absorbed (CNWBOND Carbon-GCB SPE Cartridge, 250mg, 3ml; ANPEL, Shanghai, China, www.anpel.com.cn/cnw) and filtrated (SCAA-104, 0.22 um pore size; ANPEL, Shanghai, China, http://www.anpel.com.cn) before UPLC-MS/MS analysis.

Metabolite quantification was carried out using multiple-reaction monitoring (MRM) mode. Quality control analysis was carried out by overlapping the display of Total Ion Chromatography (TIC) from the different samples detected by mass spectrometry. Principal Component Analysis (PCA) and Partial Least Squares-Discriminant Analysis (PLS-DA) were used to maximize the metabolome difference between those samples. Fold change and variable importance in project (VIP) were

used to screen differential metabolites. Metabolites with VIP>1 and fold change>2 or fold change<0.5 were defined as differentially accumulated metabolites between compared samples[38-42].

RESULTS

Detection of metabolites according to LC-MS/MS. In order to study the differences and changes of metabolites in *B. napus* seeds, UPLC-ESI-MS/MS was used. At the stage of data quality control, total ion current (TIC) maps of different quality control samples were overlapped and analyzed by mass spectrometry. The results revealed that the overlap of the QC sample detection curves was very high, which indicates that the repeatability and reliability of the mass spectrometry data was also high (Figure S1A and S1B). 726 metabolites were identified by widely targeted metabolomics. There were differences in the contents of metabolites between OA and HOA, but no special metabolites were found in any one variety. The top three metabolites identified were organic acids and derivatives (113 species), amino acids and derivatives (94 species) and lipids (75 species) (Figure S1C).

To improve the analysis of these metabolites, principal component analysis (PCA) was used to normalize the data. PC1 represents the most obvious features in the multidimensional data matrix, and PC2 represents the most relevant features in the data matrix except PC1. According to the principal component analysis of 2 samples and 3 biological repeats of quality control samples, it was observed that there was a clear distinction between OA and HOA-T3. The first principal component and the second principal component under different characteristics were 47.62% and 11.33% (Figure S1D). These results show that the inhibition of *FAD2* expression was the main reason for the differences in the metabolites of the samples. Pearson correlation coefficient R was used as the evaluation index of biological repetitive correlation, with R2 values

close to 1(Figure S1E). The metabolite data obtained were relatively accurate and reproducible, which could then be used for follow-up analysis.

Metabolic profile differential analysis of the *B. napus* seed between OA and HOA. In this study, based on partial least squares discriminant analysis, there were 112 metabolites (24 down-regulated and 88 up-regulated) between HOA-T3 and OA (Figure 1A). In addition, we also found that the results of KEGG annotation of significant differential metabolites were classified according to the type of pathway in KEGG, and the highest number of biological pathways were plant metabolic pathways and biosynthesis of secondary metabolites (Figure 1B). Purine metabolism, phenylalanine metabolism and glyceride metabolism revealed more significance by KEGG enrichment analysis (Figure 1C). However, the acyl fatty acids that provide acyl substrates for glycerol assembly are significantly different from those in OA. In general, the silencing of *FAD2* expression caused a series of changes in metabolites in *B. napus* seeds. However, the liposome library identified by widely targeted metabolomics could not meet the requirements of *B. napus* seed lipid molecular identification, so it was necessary to further identify and analyze the seed lipids.

Lipids profile differential analysis of *B. napus* seed between OA and HOA. Many changes in lipid profiles existed between the two seeds. Firstly, the relative intensity of carbon atoms C18:1 and C18:2 in species related to TAGs synthesis was investigated. The Dels of HOA C18:1 in different generations were significantly up-regulated relative to OA, whilst the Dels of C18:2 were significantly down-regulated (Figure 2). Due to the large accumulation of oleic acid, glycolipids and phospholipids

231 composed of two fatty acids also showed consistency compared with OA, but a small
232 part of them varied from generation to generation (Figure 3 and Figure 4). At the same
233 time, the contents of other long-chain fatty acids (C16, C20) also changed, indicating
234 that high OA varieties with silenced *FAD2* expression completely changed the original
235 balance of biosynthesis and storage of different internal lipid molecular species.

236 **Analysis of Expression of Genes Related to the Kennedy Pathway.** The relative
237 expression of the *FAD2* gene and Kennedy pathway related genes were detected by
238 real-time qPCR. The results showed that *FAD2*, *FAD3* and *TGD1* genes were
239 significantly down-regulated in HOA, whilst *LPAT2* and *DGAT2* genes were up-
240 regulated (Figure 5). The results of real-time qPCR showed that the intron-containing
241 hairpin RNA (ihpRNA) plant expression vector may well inhibit the expression of the
242 *FAD2* gene in each generation of HOA. A potential factor may be that the expression
243 of genes related to the Kennedy pathway also changed the feedback regulation, possibly
244 due to a change in metabolites initiated by the accumulation of OA.

245 **Analysis of *B. napus* seed germination between OA and HOA**. The inhibition
246 of *FAD*2 expression resulted in many changes in metabolites and the expression of
247 genes. Some of the pathways were related to plant growth and development, so it was
248 necessary to understand the changes of *B. napus* seed germination. The germination
249 potential of each sample was between 79.7% to 88.3%, and the order of germination
250 potential was WT>T4>T3>T9>T7. The germination rate of each sample was between
251 80.0% to 89.0%, and the order of germination rate was WT>T4>T3>T9>T7. The same
252 lowercase letters showed no significant difference at $P<0.05$ level, and the same

uppercase letters showed no significant difference at P<0.01 level (Table 1). There was no significant difference in germination potential and germination rate among all samples, indicating that the silence of *FAD2* did not affect seed germination.

Analysis of *FAD2* Gene structure in HOA by whole genome resequencing. Through analysis of the flanking sequence PCR product electrophoresis map (Figure S2A), the ihpRNA plant expression vector was integrated into the *B. napus* genome and inherited stability for many generations. Sequencing verified the consistency of the sequence (Figure S2B). Through blast, it was observed that the insertion site might be on chromosome A09 of the *B. napus* genome (Figure S2C). Whole genome resequencing revealed that distribution statistics of variants T3, T9 and OA on the genome were roughly the same (Figure 6A, 6B and 6C). SNP results of T3 and T9 generations filtered by OA showed that the variations on each chromosome of the two transgenic generations were almost the same, and there was no significant difference in the distribution of each region of the chromosome. The SNP variations were mainly concentrated in chromosome C03, whilst there was a small variation in A04, A09 and C07 (Figure 6D and 6E). The InDel results of T3 and T9 generations filtered by WT showed that the variations on each chromosome of the two transgenic generations were almost the same, and there was no significant difference in the distribution of each region of the chromosome. The InDel variations were concentrated in part of A04, A09, C03 and C07(Figure 6F and 6G). The genomic distribution regions of SNP and InDel were also similar after filtering by WT (Figure 6H and 6I). The same results were obtained by whole genome resequencing insertion site analysis. The insertion site was

not in the coding gene region. Compared with OA and HOA in different generations, we found that there were no genomic changes in the *FAD2* gene, and this demonstrated that the decrease of *FAD2* expression was caused by the introduction of the iphRNA vector.

DISCUSSION

Brassica napus L. is one of the most important oil crops and the major source of vegetable oil in China[43]. Compared with polyunsaturated fatty acids (linoleic acid, linolenic acid), oleic acid has additional stable chemical properties and is not easy to oxidize. High OA rapeseed oil has a longer shelf life and can reduce the content of low-density lipoprotein in blood vessels and effectively prevent cardiovascular disease. High OA rapeseed oil is inexpensive, and its fatty acid composition and function are similar to high-priced olive oil, so it can be used as a substitute to increase economic value. Although many studies have been carried out on the yield and quality of *B. napus* seed, the effects of increased OA content on other metabolites is not clear. Changes in these metabolites is of significance to the genetic improvement of *B. napus* seed. In this study, we used widely targeted metabolomic methods to identify significant differences in seed metabolites from two genotypes of *B. napus.* A total of 726 metabolites were accurately quantified, of which 112 metabolites were significantly different (24 down-regulated and 88 up-regulated). Metabolites associated with purine metabolism, phenylalanine metabolism and glyceride metabolism were significantly different, indicating that seed-specific silencing of *FAD2* expression can cause changes in many metabolites.

Further comparative analysis of liposomes was conducted to study the changes of glycolipids and phospholipids in the seeds. Many DELs were accurately quantified, in which C18:1 was significantly up-regulated compared with OA, whilst C18:2 was significantly down-regulated. One possible reason was that silencing of *FAD2*

expression lead to a large accumulation of OA (C18:1), which had a negative effect on the substrate of C18:2. The up-regulation of phospholipids and glycolipids by C18:l also showed consistency compared with OA, but a small proportion varied from generation to generation, and this requires further experimental study. The contents of other long-chain fatty acids (C16, C20) also changed, indicating that high OA varieties with silenced *FAD2* expression completely changed the original balance of biosynthesis and storage of different internal lipid molecular species.

Rapeseed oil obtained from *B. napus* seed, is different from linoleic acid and linolenic acid, as oleic acid is relatively stable and has the valuable characteristic of reducing blood cholestrol[44]. Epidemiological studies have shown that residents in Mediterranean countries live longer and have lower rates of cardiovascular disease and breast cancer, which is closely related to the widespread consumption of olive oil rich in oleic acid[45]. Therefore, reducing the content of polyunsaturated fatty acid in edible rapeseed oil and increasing the content of oleic acid is one of the important goals of rapeseed quality breeding. Previous studies have shown that the accumulation of 18:1-acyl will inhibit the activity of plastid acetyl-CoA carboxylase (ACCase) in *B. napus*[46]. At the same time, seed germination at low temperature was adversely affected by the inhibition of *FAD2* expression[47], so we calculated the germination potential and germination rate of OA and several generations of HOA seeds, and found that there was no significant difference among the samples. High OA *B. napus* seed transformed the seed-specific expression promoter Napin and did not inhibit *FAD2* expression at other stages of plant growth.

After studying the effect of the ihpRNA plant expression vector on genomes, we determined that the vector was inserted in the non-coding region of chromosome A09. For genome-wide resequencing, distribution statistics for OA and HOA differences on chromosomes were roughly the same, whilst the InDel and SNP of T3 and T9 filtered by OA had almost no difference on each chromosome. Expression *FAD2* was significantly down-regulated in seeds from each generation, and the gene sequence did not change revealing that HOA was an effective seed variety. Expression of genes related to the Kennedy pathway also changed, and feedback regulation caused by the accumulation of OA was a potential factor. The relationship between the expression of these genes and metabolites needs to be investigated further.

As a result of this research, we found that the ihpRNA plant expression vector targeting *FAD2* gene increased the content of OA and decreased the content of linoleic acid in *B. napus* seed. It did not produce harmful substances to humans or the environment, whilst the seed-specific promoter made RNAi only occur at the seed stage. Higher quality rapeseed oil could be obtained without adversely affecting the growth and development of the plant. This may be a potential way to obtain high OA rapeseed oil through seed specific low expression of FAD2 compared to genome editing of FAD2. By studying the effect of RNAi on the metabolites of *B. napus* seed, this investigation has not only helped to improve our understanding of the process of oil accumulation, but also, to provide guidance for future breeding programs in the production of high OA seeds.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: XXXXXXXX.

Figure S1 Quality control analysis and Repetitive correlation assessment**. Figure S2**Analysis of insertion site. **Table S1** List of primers used for qRT-PCR experiment.

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Notes

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Figure Legends

Figure 1. Differential metabolite screening and Functional annotation and enrichment analysis of differential metabolites by KEGG.

(A)Volcano map of differential metabolite by VIP and fold change. Each point in the volcano map represents a metabolite, the Abscissa represents the logarithm of the multiple of quantitative difference between the two samples, and the ordinate represents the VIP value. The greater the Abscissa absolute value, the greater the expression multiple difference between the two samples; the greater the vertical coordinate value, the more significant the differential expression, and the more reliable the screened differentially expressed metabolites. The green dots in the picture represent down-regulated differentially expressed metabolites, red dots represent up-regulated differentially expressed metabolites, and gray represents metabolites detected but not significantly different.

(B)KEGG taxonomic diagram of differential metabolites. The ordinate is the name of the KEGG metabolic pathway, and the abscissa is the number of metabolites annotated to the pathway and its proportion to the total number of metabolites annotated.

(C)Bubble diagram of KEGG enrichment of differential metabolites.

Figure2. Analysis of the content of Lyso-phospholipids.

LPC,Lyso-phosphatidylCholine.LPE,Lyso-Phosphatidylethanolamine.LPG,Lyso-phosphatidylglycerol.WT,Wild type *Brassica napus* Westar.T3,T4,T7,high OA

Brassica napus W-4.The data shown were the average mean±standard error (SE) of three replicates (n=3),Significant differences were determined by one-way analysis of variance (ANOVA)(*P<0.05,**P<0.01).

Figure 3. Analysis of the content of Phospholipid.

(A)The content of Phosphatidyl serines.

(B) The content of Phosphatidyl ethanolamines.

(C) The content of Phosphatidyl glycerol.

(D) The content of Phosphatidic acid.

(E) Th econtent of Phosphatidyl inositols.

(F) The content of Phosphatidyl cholines.WT,Wild type *Brassica napus* Westar.T3,T4,T7,high OA *Brassica napus* W-4.The data shown were the average mean ±standard error (SE) of three replicates (n=3),Significant differences were determined by one-way analysis of variance (ANOVA)(*P<0.05,**P<0.01).

Figure4. Analysis of the content of Glycolipid.

(A) The content of Digalactosyl diglyceride.

(B) The content of Monogalactosyl diglyceride.WT,Wild type *Brassica napus* Westar.T3,T4,T7,high OA *Brassica napus* W-4.The data shown were the average mean ±standard error (SE) of three replicates (n=3),Significant differences were determined by one-way analysis of variance (ANOVA)(*P<0.05,**P<0.01).

Figure 5. Expression profile of *B.napus*kennedy pathway related genes in WT and W-4.

(A-D) *Bnfad2*(A), *Bnlpat2*(B),*Bndgat2*(C),*Bnfad3*(D) and*Bntgd1*(E).*Bnactin7* was amplified as an internal control.The data shown were the average mean±standard error (SE) of three replicates (n=3),Significant differences were determined by one-way analysis of variance (ANOVA)(*P<0.05,**P<0.01).

Figure 6. Difference analysis of genome-wide resequencing.

(A)Distribution of structural variation of genome of W-4 T-3.

(B)Distribution of structural variation of genome of W-4 T-9.

(C)Distribution of structural variation of genome of WT. From outside to inside, the order is: Chromosome, SNP,InDel,CNV duplication,CNV deletion,SV insertion,SV deletion,SV invertion (SV ITX, SV CTX).

(D)Heat map of density distribution of SNP filtered by WT in W-4 T3 on each chromosome.

(E) Heat map of density distribution of SNP filtered by WT in W-4 T9 on each chromosome.

(F)Heat map of density distribution of InDel filtered by WT in W-4 T3 on each chromosome.

(G)Heat map of density distribution of InDel filtered by WT in W-4 T9 on each chromosome.

(H)Distribution of SNP filtered by WT in W-4 T-3 and T-9 in different regions.

577 (I)Distribution of InDel filtered by WT in W-4 T-3 and T-9 in different regions.

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