Transcriptomic analyze of the less branches of *Brassica napus* L. suitable for mechanized harvesting

Yiren Qing, Yaoming Li* and Zheng Ma

Key Laboratory of Modern Agricultural Equipment and Technology, Ministry of Education, Jiangsu Province, Zhengjing, Jiangsu, China

*corresponding author’s e-mail: yml@ujs.edu.cn

**Abstract.** Rapeseed (*Brassica napus* L.) is one of the main oil crops in the world. Its multi branching characteristics cause great difficulties and losses to mechanical harvesting. Less branched plant type is a favorable feature for promoting mechanical harvesting. In this paper, a natural mutant rape of less branches with NY12 (Ningyou 12) as background was obtained, which was named *sfz* (shao fen zhi, meaning less branches in Chinese). The mutant showed a significant decrease in branching and a significant increase in the lowest branch height, which are beneficial to harvest. Transcriptomic analysis of shoot tip tissue showed that the expression of genes related to strigolactone is up-regulated, which promotes its inhibitory effect on plant branching, and the expression of genes related to cell division cycle and cytoplasmic division were all down regulated. It is speculated that the decrease of cell division may also result in the decrease of branching. The research provides a basis for the formation of branching and promoted the development of mechanized harvesting.

1. **Introduction**

Rapeseed (*Brassica napus* L.) is one of the main oil crops in the world, which plays an important role in edible and industrial. But the rapeseed is difficult to harvest mechanically owing to its high plant type and multi-branched. When combined harvest the vertical cutter shearing the cross branches leading to massive loss. Some researches indicated that the plant with slender stem, higher branch starting height and fewer branches are more suitable for mechanized harvesting [1,2]. Therefore, multi-branched is the main factor affecting harvest performance, the research on the branching and promoting the development of less branched of rape are significant in promoting mechanical harvesting.

Shoot branching is comes from the axillary meristem (AM), and AM is derived from the shoot apical meristem (SAM), and its development is regulated by genetics, plant hormones and environmental signals. Branching process generally includes the initiation and the outgrowth of AM, many genes involved in regulating various stages of branching patterns development have been identified from studying several species of mutants have altered branch numbers. Among these genes, affecting AM initiation have lateral suppressor (*LS*, tomato), homologous *LAS* (Arabidopsis thaliana) and *MONOCULM1* (*MOCI*, rice), which are whole members of the GRAS family transcription factor. GRAS protein family is unique to plants and has significant function in regulatory signaling and growth [3-5]. In addition, *RAX* (*REGULATOR OF AXILLARY MERISTEMS1*, Arabidopsis thaliana) homologous to *BL* (*BLIND*, tomato), which encoded Myb family transcription factors acting in the early stage of axillary meristem formation [6-8]. In *Arabidopsis*, *CUP SHAPED COTYLEDONS (CUC1-CUC3)* encode transcription factors containing NAC domain and determines...
the establishment of leaf axilla [9], and REVOLUTA (Rev) belongs to HD-ZIP (homeodomain-leucine zipper), the mutant of defective Rev caused organ can’t produce AM in axillary [10]. LAS operate upstream of REV, while RAX plays a role through CUC2, both of which act on STM to control AM formation. TB1 gene in maize is associated with AM formation and growth, orthologous OsTB1/FINECULMI in rice and BRANCHED1 (BRC1) in Arabidopsis, overexpression of these genes inhibits branching, which is a negative regulator of branching control. In rice MOCI regulates the tillers by affecting its downstream genes OR1ZA SATIVA HOMEobox 1 (OSH1) and OsTB1 [11-15]. Hormones and key branching genes coordinate with each other to regulate plant shoot branching. More and more studies suggested that hormone signaling may also be involved in the regulation of axillary meristem formation, the SPS (supershoot) and Bus (Bushy) genes in Arabidopsis thaliana encode cytochrome P450 can regulate hormone levels, which in their corresponding mutants, the number of meristematic tissues formed in the axilla of leaves and cytokinins increased [8,9].

Moreover, many species related to AM outgrowth have been investigated, includes ramosus (rms, Pisum sativum pea), more axillary growth (max, Arabidopsis), decreased apical dominance (dad, petunia) and high tillering dwarf/dwarf (htd/D, rice), which were all multi-branched mutant and the mutation of these genes do not affect AM formation but relieve dormancy of axillary buds [16-21]. Physiological studies have shown that axillary bud growth and dormancy are regulated by hormone levels, in which the main determinants are auxin, cytokin (CK) and the strigolactone (SL) discovered recently. Polar auxin transport (PAT) represses axillary bud growth, while CK promotes it, but the bud outgrowth depends on the ratio of these two hormones rather than their absolute content level. SLs transporting from bottom to top can inhibit bud growth to control branch branching with IAA and CTK synergistically. IAA synthesized in apical buds may inhibit the expression of gen ISOPENTENYL TRANSFERASE (IPT) which synthesizes CK in lateral buds to make lateral buds dormant. PINFORMED1 (PIN1) encodes PIN-FORMED auxin efflux carriers (PIN), the expression of PIN in max mutant increased significantly exhibits SLs can suppress branching via regulating the transport of IAA [22-26]. SL is a kind of plant secondary metabolites derived from carotenoids, three enzymes are known to be involved in SLs synthesis, carotenoid cleavage di-oxygenase 7(CCD7), carotenoid cleavage di-oxygenase 8(CCD8) and cytochrome(Cyt450). Genes MAX3/RMS5/D17/HTD1 and MAX4/RMS1/DAD1/D10 encoded CCD7 and CCD8 respectively, and Cyt450 encoded by MAX1 acts downstream of MAX3 and MAX4 [27-30]. Besides MAX2, RMS4 and DWARF3 participate in SLs signal perception [31].

So far the regulation mechanism of plant branching is not understanding completely in that the insufficient of mutants. This paper we found a rapeseed natural mutant of NY12 (Ningyou 12) background that favorable for mechanized harvesting, which performed less branching and was named sfz (shao fen zhi, which means less branching in Chinese). The phenotypic characteristics of sfz and NY12 were analyzed and compared and RNA sequencing (RNA-seq) was adopted to find the differentially expressed genes between them in SAM. At the molecular level, it provides a new research basis for the formation of branching in B. napus and promoted the breeding of cultivation of mechanized varieties.

2. Materials and methods

2.1 Plant materials and sampling

B. napus cultivars NY12 and its mutants sfz were grown under normal conditions in the experimental field of Jiangsu University with 30 cm row spacing and 25 cm plant spacing on September 30, 2017, provided by Rapeseed Research Group, Academy of Life Sciences of Jiangsu University. Three replicates of shoot apexes samples of NY12 and sfz were collected at the shoot apexes just bolted on January 8, 2018, which one sample from three individual shoot apexes mixed for transcriptomic analysis. All tissues were immediately frozen in liquid nitrogen after gathered and then stored in a refrigerator at -80°C before use.
2.2. Evaluation of agronomic traits and data analysis
In maturation period, 15 plants were selected randomly from NY12 and sfz mutants for traits statistics. Some phenotypic characteristics associated with mechanized harvesting were investigated, including plant height, stem diameter of main stem, primary branching number, lowest branch height (height from cotyledon node to the lowest branch), relative length of branched stem (the ratio of length of stem with branches to plant height), main inflorescence length (the length between the lowest and the highest pod of the main stem), effective pods number of main stem, thousand seed weight and per plant yield. These agronomic traits were measured according to the descriptors and Data Standard for Rapeseed (Brassica spp.). The analysis of statistical result were carried out by GraphPad Primer6 and drawing the charts.

2.3. RNA extraction and qualification
Total RNA of shoot apexes with sfz mutant and NY12 were extracted using Trizol reagent (Life Technologies, New York, USA), quality of purified RNA was verified by Nanodrop and NanoDrop 2000(Thermo) and further an accurate detection using the RNA Nano 6000 Assay Kit of the Agilent Bioanalyzer 2100 system (Agilent Technologies, CA, USA).

2.4. Library Construction and sequencing
After extracting the total RNA of the sample, a total amount of 1 μg RNA per sample was used as input material for the RNA sample preparations. Sequencing libraries were generated using NEBNext UltraTM RNA Library Prep Kit for Illumina (NEB, USA) according to the manufacturer’s recommendations. To obtain clean data from raw data by removing reads containing adapter, reads containing ploy-N and low quality reads.

2.5. Gene annotation and bioinformatics analysis
Then clean reads were mapped to the reference genome sequence (http:// www. genoscope. cns.fr /brassicanapus/data/). Only reads with a perfect match or one mismatch were further analyzed and annotated based on the reference genome. Tophat2 tools soft were used to map with reference genome. Gene function was annotated based on the following databases: Nr (NCBI non-redundant protein sequences); Nt (NCBI non-redundant nucleotide sequences); Pfam (Protein family); KOG/COG (Clusters of Orthologous Groups of proteins); Swiss-Prot (A manually annotated and reviewed protein sequence database); KO (KEGG Ortholog database); GO (Gene Ontology). NCBI database was used to annotate gene function.

2.6. Differential expression analysis
Differential expression analysis of two groups was performed using the DESeq R package for the samples with biological replicates [32]. Differentially expressed genes (DEGs) were identified with the threshold for was |log2(fold change)| ≥ 1 and FDR(False Discovery Rate) < 0.001. Fold Change represents the ratio of expression between two samples. FDR is obtained by correcting p-value of difference significance using the Benjamini and Hochberg’s approach.

2.7. GO enrichment analysis
Gene Ontology (GO) enrichment analysis of the differentially expressed genes (DEGs) was implemented by the GOseq R packages based Wallenius non-central hyper-geometric distribution (Young et al, 2010), which can adjust for gene length bias in DEGs.

2.8. KEGG pathway enrichment analysis
Take pathway in the KEGG (Kyoto Encyclopedia of Genes and Genomes) database as the unit, apply hypergeometric test to find the pathway that is significantly enriched in DEGs compared with the background of the whole genome.
3. Results

3.1. Phenotypic characterization analysis of sfz mutant
The sfz mutant was discovered in experimental field of wild type NY12 background and it was self-crossed for more than 5 years to obtain the homozygote with no segregation of character. Compared to wild type NY12, sfz exhibited many phenotypic characteristics which were beneficial to mechanized harvesting. At flowering and maturing stages, the number of primary branches of sfz mutants is significantly less than the wild-type NY12 (Figure 1a, b). At flowering stage, the mutant sfz had fewer branches than wild type, and in the maturity, the branches of the mutant sfz were more concentrated, the main inflorescence was more obvious, and the pod maturity was more consistent. Too many primary branching number result in branches sagging intertwined and pods maturation period inconsistent seriously, which is very difficult for vertical cutter to divide and cut grain in mechanized harvesting, causing serious header loss.

The statistical agronomic traits of wild type and mutant sfz are shown in the Table 1, in which, the abbreviations are as follows: PH (plant height), MSD (Main stem diameter above ground 20cm), PBN (primary branching number), LBH (lowest branch height), RLBS (relative length of branched stem), MIL (main inflorescence length), PNMI (pods number of main inflorescence), TSW (thousand seed weight) and PPY (per plant yield). It can be seen that except for the PH, TSW and PNMI, the remaining traits are significantly different between the wild type and mutant. The main stem diameter above ground 20cm of sfz was thinner than NY12, it is advantageous to cut for harvester so that to reduce vibration and loss. In addition the lowest branch height of sfz increased markedly while the relative length of branched stem was substantially smaller than NY12, which imply that branching is relatively concentrated, that is to say there are fewer staggered parts of rapeseed plants. The above characteristics is conducive to reduce branching vibration and can meet the requirements for energy in late growth stage of rapeseed. However, the per plant yield of sfz is less than NY12, yet, the less branching characteristic of sfz is more conducive to close planting, and proper close planting can make up for the lack of yield and is more conducive to harvesting.

![Figure 1. Comparison of plant type characteristics between wild type and mutant wild type NY12 (left) and sfz mutant (right). (a) The mutant sfz had fewer branches at flowering stage compared to NY12, Bar=20 cm; (b) The mutant had more concentrated branches and more consistent pod maturity at maturity, Bar=20 cm.](image-url)
Table 1. Comparison of agronomic characters between wild type NY12 and mutant sfz. Lowercase letters indicate that the pod volume is significantly different in one line based on Fisher’s protected LSD (P=0.05).

| Type   | PH (cm) | MSD (mm) | PBN | LBH (cm) | RLBS | MSL (cm) | PNMI | TSW (g) | PPY (g) |
|--------|---------|----------|-----|----------|------|----------|------|--------|---------|
| sfz    | 186.46a | 14.97b   | 7.19b | 77.73a   | 0.20b| 69.93b   | 62.00a| 3.59a  | 16.32b  |
| NY12   | 184.62a | 16.67a   | 10.23a| 65.77b   | 0.38a| 47.08a   | 74.83a| 3.24a  | 22.15a  |

3.2. Gene differential expression analysis
High-throughput RNA-seq of sfz and NY12 was carried out to explore the underlying reason mutation phenotype of sfz at transcriptional level. After quality control of sequencing, the Clean Data of each sample reached 6.71 Gb, and the percentage of Q30 base in each sample was not less than 93.44%. The relative efficiency Clean Reads mapped to the reference genome was 73.33%~75.22% by the Tophat (v2.0.12). In order to obtain reliable differentially expressed genes(DEGs), three biological repeats were established every group, the Pearson’s Correlation Coefficient of repeated samples were calculated entirely greater than 0.9. A total of 64998 genes were annotated, 8084 significant DEGs were identified compared to wild type. Volcano map of differential expression genes are shown in Fig. 2, among, 3317 genes of which were up-regulated and 4767 were down-regulated.

3.3. Expression level of candidate genes from the transcriptomic data
GO (Gene Ontology) functional classification of 8084 DEGs was performed to make further understanding of gene function at molecular level, in which 6,757 genes were successfully annotated. Enrichment analysis of biological processes (BP), cell components (CC) and molecular functions (MF) were carried out, the GO classification statistics of DEGs are shown in the Fig. 3. The results shows that the three classifications have enriched 19, 14 and 11 secondary functions respectively. In biological process, the top three categories with the largest proportion of genes were cell process, metabolic process and single-organism process; and DNA binding, catalytic activity and nucleic acid binding transcription factor activity in molecular function category. These results indicated that DEGs are mainly involved in gene expression regulation and stress response, function as a structural molecule and DNA binding. For biological process, the special significantly enriched terms includes “GO:0016572 histone phosphorylation”, “GO:0051567 histone H3-K9 methylation”, “spindle assembly”, “GO:0008017 microtubule-based movement”, “GO:0010389 regulation of G2/M transition of mitotic cell cycle” and “regulation of DNA replication”, “negative regulation of gene expression, epigenetic”; and for MF, “glutathione peroxidase activity”, microtubule binding, microtubule motor activity, cellulose synthase (UDP-forming) activity. In the enrichment analysis of KEGG pathway, only “Other glycan degradation” and “Photosynthesis - antenna proteins” were significantly enriched.
4. Discussion

Plant branching regulation model as shown in Fig. 4. By analysis of the differentially expressed genes annotation, we found a gene required for lateral shoot meristems and flower meristems initiation, the Rev, was down-regulated, and the MAX2 act as responder to strigolactones expressed up-regulated. Meanwhile, many other genes related to auxin and cytokinin signal transduction also varied obviously in sfz SAM. We know auxin response factors (ARFs) are transcription factor, and Aux/IAAs as early auxin response genes could interact with ARFs proteins forming heterodimers to modulate early auxin response genes expression. In this study, ARFs (ARF1, ARF2 and ARF3) were down-regulated, however, most IAAAs include IAA11, IAA16, IAA18, IAA26 and IAA28 were up-regulated except IAA9. About Polar Auxin Transport (PAT), PID kinase acts as a positive regulator of cellular auxin efflux performed up-regulated, instead, ABCB19 as for auxin efflux transporter was down-regulated and ABCB4 as auxin influx transporter expressed up regulation. In cytokinin signal transduction, the Arabidopsis histidine kinases2 (AHK2) as cytokinins (CK) receptor exhibited up-regulated in sfz mutant. In addition, all the DEGs with the annotation of type A-ARABIDOPSIS RESPONSE REGULATORS (ARRs) (ARR4, ARR6 and ARR9) were up-regulated in sfz SAM. For type B-ARRs, ARR14 was down-regulated, on the contrary, ARR12 showed an up-regulation of expression. In general, the expression of negative cytokinin regulator ARR-A was increased, while that of positive cytokinin regulator ARR-B was decreased, which inhibited the promoting effect of cytokinin on plant branching. Furthermore, gene TIFY 11B as for repressor of jasmonate responses were up regulation.

In conclusion, this paper verifies that strigolactone does have an inhibitory effect on plant branches. In addition, in the sfz mutants, we found that the expression of genes related to cell division cycle and cytoplasmic division were all down regulated. Therefore, we speculated that slow cell division might be another reason for the decrease of branching.
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