Proteomic analysis of a clavata-like phenotype mutant in *Brassica napus*

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Abstract

Rapeseed is one of the important oil crops in China. Better understanding of the regulation network of main agronomic traits of rapeseed could improve the yielding of rapeseed. In this study, we obtained an inflorescence mutant that showed a fusion phenotype, similar with the *Arabidopsis clavata-like* phenotype, so we named the mutant as *Bnclavata-like* (*Bnclv-like*). Phenotype analysis illustrated that abnormal development of the inflorescence meristem (IM) led to the fused-inflorescence phenotype. At the stage of protein abundance, major regulators in metabolic processes, ROS metabolism, and cytoskeleton formation were seen to be altered in this mutant. These results not only revealed the relationship between biological processes and inflorescence meristem development, but also suggest bioengineering strategies for the improved breeding and production of *Brassica napus*.

Keywords: *Brassica napus*, proteomic, inflorescence meristem (IM), Bnclavata-like (*Bnclv-like*), quantitative real-time PCR.

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Introduction

As one of the four greatest oil crops in the world, *Brassica napus* L., plays a crucial role in world oil crops. First of all, rapeseed is an essential organic material for edible oil, and it is rich in fatty acids (such as linoleic acid, linolenic acid). Secondly, rapeseed meal is rich in protein, which is a potential source for the feed protein. Meanwhile, rapeseed stalks, like wheat and maize, can also be used as raw materials for the production of new bio-energy and as an important energy crop. Rapeseed is also a great source of nectar and ornamental plants (Wang et al., 2016). Because the three components of inflorescence structure (number of siliques per plant, number of seeds per siliques and 1000-seed weight) is closely correlated with the seed yield in rapeseed, the discovery of optimal inflorescence structure will be helpful to improve the productivity of rapeseed (Chen et al., 2007; Lu et al., 2017; Zhang et al., 2018).

Inflorescence development affects plant morphogenesis, yield and quality. Studies in *Arabidopsis thaliana* and rice have demonstrated that transcription factors and hormones play a significant role in inflorescence development and lateral branching regulation (Hofmann, 2009; Bongers et al., 2014; Chew et al., 2014; Leduc et al., 2014; Cai et al., 2016; Li et al., 2017; Wang and Jiao, 2018). In *Arabidopsis thaliana*, TERMINAL FLOWER 1 (*TFL1*), LEAFY (*LFY*) and APALA 1 (*API*) are characteristic genes of the floral meristem, and their antagonistic interactions can regulate inflorescence branching patterns (Ma et al., 2017). *TFL1* was specifically expressed in main inflorescence meristem and lateral inflorescence meristem, while *LFY* and *API* were abundantly asserted in the floral meristem (Winter et al., 2015). The *TFL1* loss-of-function mutant may cause heterotopic expression of *LFY* and *API* genes, contributing to the transformation of inflorescence meristem into floral meristem, precious flowering formless inflorescence branching in *Arabidopsis thaliana*. On the contrary, overexpression of *TFL1* in *Arabidopsis thaliana* could inhibit the expression of *LFY* and *API*, and thus delay flowering and increase inflorescence branching (Cheng et al., 2018). *API* protein and its homologs CAULIFLOWER (CAL) and FRUITFULL (FUL) in *Arabidopsis thaliana* could inhibit the expression of *TFL1* gene (Parey et al., 2002), while *LFY* protein can promote the expression of *TFL1* gene (Serrano-Mislata et al., 2017). *Arabidopsis ARGONAUTE1* (*AGO1*) could also inhibit the expression of *TFL1* gene and regulate inflorescence development (Fernandez-Nohales et al., 2014). SHORT VEGETATIVE PHASE (*SVP*), SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1 (*SOC1*), AGAMOUS-LIKE 24 (*AGL24*) and SEPLLATA 4 (*SEP4*) belong to MADS-box transcription factors, which could regulate flowering time and directly inhibit the expression of *TFL1* in newly floral meristem, and thus regulate inflorescence development (Liu et al., 2013).

Phytohormones, especially auxin (IAA) and cytokinin (CK), are key regulators of inflorescence structure, playing an important role in inflorescence growth and development.
Arabidopsis thaliana biosynthesis. There are nine cytokinin-activating enzyme, catalyzes the last step of CK biosynthesis pathway by inhibiting the expression of some auxin biosynthesis genes, such as YUCCA1 (YUC1) and YUCCA4 (YUC4) (Moyroud et al., 2011; Li et al., 2013; Winter et al., 2015). However, LFY gene could promote the expression of PINOID (PID), an auxin transport regulator (Yamaguchi et al., 2013). In Arabidopsis, cytokinins promote inflorescence meristem development and affect inflorescence structure by promoting the expression of WUSCHEL (WUS) gene and inhibiting the expression of CLAVATA1 (CLV1) and CLV3 (Gordon et al., 2009). LONELY GUY (LOG), encoding a cytokinin-activating enzyme, catalyzes the last step of CK biosynthesis. There are nine LOG homologous genes in Arabidopsis thaliana. The log3, log4, log7 triple mutant and log1, log2, log3, log4, log5, log7, log8 seven-mutant produce fewer floral meristem, indicating that the development of inflorescence meristem requires CK (Kuroha et al., 2009; Tokunaga et al., 2012). In Arabidopsis, AP1 could reduce the expression level of CK biosynthesis gene LOG1, but activate the cytokinin-degrading gene CKX3 by binding directly to the promoter of the target gene (Ma et al., 2017; Joshi et al., 2018). The study in Arabidopsis showed that mutations in AHK2, AHK3 and AHK4, which encodes CK receptor histidine kinase, reduced inflorescence stem length (Nishimura et al., 2004).

Optimized inflorescence architecture is fundamental for high-yield breeding of rapeseed. Thus, much research has been done on the genetic mechanism of inflorescence structure (Cai et al., 2016; Zhao et al., 2016; Zhang et al., 2018). However, insufficient information is available on the development of rapeseed. Here, we present Bnclv-like, a natural B. napus mutant, which was characterized by abnormal development of inflorescence meristem (IM). Two-dimensional electrophoresis (2-DE) was used to reveal the mechanism of the change in protein level. The proteins involved in IM regulation displayed significant variation, which could provide molecular basis for IM development and inflorescence structure formation in Brassica napus.

Materials and Methods

Plant materials and growth conditions

In this study, B. napus plants (Bnclv-like and Ningyou 12) were grown in the experimental field of Jiangsu University. The IM samples for proteomic analysis were collected when the first flower was opening, so that the development of IMs from the mutant and the wild type could keep the same stage. All samples were frozen with liquid nitrogen immediately after harvest and stored at -80 °C before use.

Protein extraction

The total high-quality proteins from Bnclv-like mutant and Ningyou 12 (1.5 g [FW]) were extracted using the ReadyPrep protein extraction kit (Bio-Rad, USA) according to the manufacturer’s instruction with some modifications. Protein concentrations were determined using the RCDC Kit (Bio-Rad, USA) according to the manufacturer’s instruction.

Two-dimensional electrophoresis (2-DE) and image analysis

2-DE was carried out with 17 cm Immobiline DryStrips (Bio-Rad, USA, linear, pH 4-7) as using a modification of the method of Yang (Yang et al., 2014). First, 1,200 μg of total protein was loaded onto the Immobiline DryStrip using passive rehydration (12 h). Second, isoelectric focusing (IEF) was performed on an IPGphor III IEF system (GE Healthcare, USA) with these steps: at 300, 500, 1,000 and 8,000 V for 1 h each and then held at 8,000 V until a total voltage of 54,000 Vh was reached. Third, the isoelectric focused strips were equilibrated for 15 min in equilibration buffer (0.5 M Tris-HCl, pH 6.8, 2.5% SDS, 30% v/v glycerol and 1% DTT) and then equilibrated again for 15 min (0.5 M Tris-HCl, pH 6.8, 2.5% SDS, 30% (v/v) glycerol and 2.5% (w/v) iodoacetamide). Fourth, second-dimensional electrophoresis was done with a Laemmli buffer system using 5% stacking gels and 15% resolving gels. At last, the gels were stained with 0.116% Coomassie brilliant blue R-250 in a solution containing 25% (v/v) ethanol and 8% acetic acid.

The 2-DE gels were scanned by ImageScanner III (GE Healthcare, USA) at transparency mode with 300 dpi resolution. Gel comparison and spot analysis were performed using ImageMasterTM 2D platinum version 7.0 software (GE Healthcare, USA) according to the manufacturer’s instruction. The intensity ratio of the corresponding spots in different gels was calculated and spots with a ratio ≥ 2 and an ANOVA ≤ 0.05 were defined as differential spots. The experiment was repeated three times with independent samples.

Mass spectrometry (MS) analysis and data analysis

The differential protein spots in Bnclv-like mutant and Ningyou 12 were excised manually from the gels and rinsed in ultrapure water with two rounds of ultrasonic treatment (10 min/each). The proteins were digested in gels according to the method of Yang et al. (2014). Then, the peptides in the resulting digestion were identified by MALDI-TOF MS (Bruker Daltonics, Ultraflex-TOF-TOF, Germany).

The database searching and protein identification of the peptide mass fingerprinting was performed as described by Yao et al. (2011). B. napus was selected as the taxonomic category. Proteins with a Mascot score > 64 were considered to be credible.

Gene ontology analysis of differential proteins

The Gene Ontology (GO) IDs of the identified proteins were obtained through InterProscan searching with the amino acid sequences and were output in txt format. Subsequently,
the annotation files of up- and down-regulated proteins and unique proteins in Bnclv-like mutant and Ningyou 12 were respectively uploaded in InterproScan.txt into WEGO (Ye et al., 2006; Ye et al., 2018). Finally, the analysis results were output as a histogram file after online operation. The protein-protein interaction network was initially constructed from differential proteins using the STRING database and reconstructed by Cytoscape.

RNA extraction and quantitative real-time PCR

To validate the differential proteins, quantitative real-time PCR (qPCR) was used to confirm the expression patterns of selected proteins in Bnclv-like and Ningyou12. The total RNA of collected samples were extracted using TRIzol reagent (Life technologies, USA) following the protocol of the supplier. First strand cDNA was synthesized by reverse transcription of total RNA (500 ng) using the HiScript Q RT SuperMix for qPCR kit (Vazyme, China). All reactions were performed with an ABI 7300 Real-Time PCR Detection System (Applied Biosystems, USA) with SYBR Green Master Mix (Vazyme, China). Primer premier 5.0 was used to design gene-specific primers according to the corresponding unigene sequences. The sequences of primers were listed in Table S1. Primers were checked for efficiency using the standard curve method, and their specificities were checked using melting curves after all qPCR runs. All qPCRs were performed in triplicate in a total volume of 20 μL. The ACTIN gene was used as an internal reference gene. The relative expression levels of genes were calculated using the 2^ΔΔCt method.

Results

Morphological and genetic characterizations of Bnclv-like mutant

We obtained a natural mutant in Ningyou 12 experimental field, which showed fused-inflorescence branching at the flowering stage (Figure 1), similar to the Arabidopsis clavata-like phenotype (Brand et al., 2000; Liu et al., 2009), therefore we named the mutant as Bnclvata-like (Bnclv-like). The Bnclv-like homozygote was obtained through self-crossing for five generations, which showed stable inheritance with no segregation of phenotypic traits was observed. Like the Bnclv-like mutant, the F1 of hybrid between Bnclv-like mutant and ZS11 (Zhongshuang 11) also exhibited the fused-inflorescence phenotype. Among 42 F2 individuals, 32 and 10 plants were identified as Bnclv-like mutant and wild-type, respectively, which fitted an expected Mendelian segregation ratio of 3:1 (χ²=0.02, P=0.90). These results indicated that Bnclv-like mutant was controlled by a dominant gene.

Protein expression profiles and differential proteins between Bnclv-like mutant and ZS11 in IM

Proteomic analysis has been widely used in the identification of various proteins in plants (Yang et al., 2014; Zhu et al., 2014; Wu et al., 2015; Yang et al., 2015; Apaliya et al., 2019). In this study, 17 cm Immobiline DryStrips (pH 4-7, linear) were used for 2-DE analysis. More than 1200 reproducible protein spots were detected in 2-DE gels (Figure 2). Fifty spots were detected to be significantly differentially expressed (ANOVA ≤0.05) (Figure 2). Relative to the wild type, 25 proteins were up-regulated and 12 proteins down-regulated in the Bnclv-like mutant. We also found 13 unique proteins in the Bnclv-like, indicating that the Bnclv-like mutation induces de novo accumulation of these proteins.

Protein identification by MALDI-TOF-MS and functional classification

After MALDI-TOF-MS analysis, 41 spots were identified successfully (Table 1). To further predict and classify the function of these proteins, the sequences of these differential proteins were used to search for genes with GO assignments. Of the 41 proteins, 37 were annotated successfully and classified to the categories of molecular function, biological process and cellular component (Figure 3A). Fifteen functional sub-categories were identified for biological process, 11 for the cellular component and 3 for molecular function. Some of the proteins were assigned to more than one sub-category. Therefore, based on the biological function of these proteins, we performed an accurate classification of the biological process (Figure 3B). The largest three sub-categories were “metabolic process”, “response to stimulus” and “cellular component organization or biogenesis”, which were essentially consistent with the results generated by BLAST2GO.

The information about metabolic pathways of the differential proteins is valuable for identifying altered physiological processes in the Bnclv-like IM. KEGG pathway analysis was performed subsequently. Twenty-one out of 37 annotated proteins were mapped to 41 biological pathways, among which “biosynthesis of antibiotics”, “glycolysis/gluconeogenesis” and “carbon fixation in photosynthetic organisms” were the three largest pathways, consisting of 11, 7 and 7 proteins, respectively (Figure 3C).

To further investigate the roles of differential proteins in the abnormal IM development in the Bnclv-like mutant, we searched for evidence of direct or functional protein-protein interactions (PPI). Based on their GO annotations, 37 proteins were chosen for PPI analysis. The results showed that

Figure 1 - Inflorescence morphology of wild type (A) and Bnclv-like mutant (B) at flowering stage. Bar=10 cm.
23 of them were predicted to interact with each other (Figure 4). In the network, TPI, GAPC1, c-NAD-MDH1, and mMDH1 were predicted to have the most interactions with other proteins. The up-regulation of TPI, c-NAD-MDH1, and mMDH1 might be a central contribution to the development of Bnclv-like IM. In addition to proteins related to metabolism, the interaction network also contained proteins involved in cytoskeleton construction and stress responses. ACT7 was up-regulated 9-fold and the expression of FSD1 was down-regulated 7-fold. In order to reveal how cytoskeletal formation and stress response proteins are related with the abnormal IM development in Bnclv-like, these two proteins were selected as the center of these two pathways to analyze the interacting networks around them. The results showed that seven proteins interacted with ACT7 (Figure 5A) and 10 proteins interacted with FSD1 (Figure 5B). Interestingly, three proteins showed interactions with both ACT7 and FSD1, indicating a connection between these two biological processes.

Quantitative real-time PCR

To confirm the accuracy of the 2-DE results, 19 genes were selected for qPCR validation (Figure S1). Fourteen genes displayed the same trend variations with the results of 2-DE, whereas five genes exhibited different directions of change in expression (Figure 6). Surprisingly, the proteins
| Spot No. | NCBI Accession No. | Description | Homolog in *A. thaliana* | MW (kDa) | pI | No. of Amino Acids | No. of Peptide matched | Seq Cover (%) | Score | Fold change |
|---------|--------------------|-------------|--------------------------|----------|----|-------------------|----------------------|--------------|-------|-------------|
| 1       | gi|9239|19510 | Chlorophyll a-b binding protein 1 | ALB3 | 28.4 | 5.33 | 267 | 12 | 32 | 375 | 2.37↑ |
| 2       | gi|6748|96987 | Profilin-1 | PRF1 | 14.1 | 4.48 | 131 | 6 | 29 | 125 | 2.71↑ |
| 3       | gi|6748|72456 | Nucleoside diphosphate kinase 1 | NDPK1 | 15.6 | 5.91 | 140 | 8 | 26 | 176 | 2.64↑ |
| 4       | gi|6749|39853 | BnaA06g05150D | BnaA06g05150D | 15.3 | 5.44 | 152 | 8 | 40 | 264 | 3.91↑ |
| 5       | gi|6748|65899 | Adenylylsulfatase HINT1 | HINT1 | 14.2 | 5.91 | 129 | 14 | 74 | 480 | 2.1↓ |
| 6       | gi|9237|85213 | 40S ribosomal protein S12-2 | Rsp12 | 15.9 | 5.54 | 146 | 8 | 36 | 244 | 2.08↓ |
| 7       | gi|9237|02651 | PLAT domain-containing 2 | PLAT2 | 20.5 | 5.15 | 182 | 14 | 33 | 430 | 2.68↑ |
| 8       | gi|4080|5177 | Eukaryotic translation initiation factor-5A-2 | FBR12 | 17.4 | 5.56 | 207 | 8 | 40 | 458 | 5.66↑ |
| 9       | gi|9239|89255 | Superoxide dismutase [Cu-Zn] | SOD2 | 21.5 | 6.79 | 230 | 8 | 16 | 193 | 2.86↑ |
| 10      | gi|9239|27649 | Elongation factor P (EF-P) family | EF-P | 25.8 | 6.74 | 212 | 8 | 11 | 162 | 7.59↓ |
| 11      | gi|9236|41432 | Fe superoxide dismutase 1 | FSD1 | 23.8 | 6.16 | 212 | 8 | 16 | 193 | 2.86↑ |
| 12      | gi|6748|81149 | NAD(P)-binding Rossmann-fold superfamily | NAD(P)-binding Rossmann-fold superfamily | 34.9 | 8.45 | 324 | 10 | 19 | 261 | 4.02↓ |
| 13      | gi|6749|29671 | BnaA02g19100D | BnaA02g19100D | 25.4 | 5.19 | 229 | 9 | 28 | 248 | 3.45↑ |
| 14      | gi|6749|01017 | Actin-7 | ACTIN7 | 39.3 | 5.2 | 353 | 18 | 32 | 476 | 9.34↑ |
| 15      | gi|9237|91605 | Ferredoxin-NADP(+) oxidoreductase 1 | FNRE | 42.7 | 8.29 | 378 | 18 | 33 | 368 | 2.44↓ |
| 16      | gi|9236|25827 | Glucan endo-1,3-beta-acidic isoform | BG2 | 37.7 | 4.78 | 340 | 18 | 26 | 352 | 2.93↓ |
| 17      | gi|6746|91653 | BnaC08g28150D | BnaC08g28150D | 36.4 | 4.7 | 329 | 20 | 35 | 443 | 3.42↓ |
| 18      | gi|9236|81807 | Glucose-6-phosphate 1-epimerase | GAPC1 | 34.1 | 5.98 | 306 | 11 | 34 | 209 | 2.49↑ |
| 19      | gi|9375|75704 | Glyceraldehyde-3-phosphate dehydrogenase | G6PD | 37.7 | 7.7 | 339 | 9 | 25 | 306 | 2.26↓ |
| 20      | gi|9236|21706 | UDP-glucose pyrophosphorylase | UGP1 | 51.8 | 5.41 | 469 | 14 | 20 | 435 | 2.24↑ |
| 21      | gi|9235|41562 | Gamma-glutamylcysteine synthetase | GSH1 | 58.3 | 6.02 | 514 | 28 | 24 | 612 | 20.97↑ |
| 22      | gi|6749|12853 | NAD(P)-dependent glyceraldehyde-3-phosphate dehydrogenase | ALD1H1A3 | 54.7 | 6.43 | 503 | 20 | 22 | 397 | 7.11↑ |
| 23      | gi|6748|88463 | Probable mitochondrial-processing peptidase subunit | MPPBET | 58.9 | 6.23 | 529 | 21 | 29 | 536 | 5.71↑ |
| 24      | gi|6748|85646 | Hsp70-Hsp90 organizing 2 | RING/U-box | 63.9 | 5.77 | 562 | 40 | 43 | 860 | 2.34↓ |
| 25      | gi|9235|41562 | Germin subfamily 3 member 3 | GER3 | 22.6 | 5.4 | 211 | 6 | 27 | 274 | 3.45↑ |
| 26      | gi|6748|68327 | Probable 6-phosphogluconolactonase chloroplastic | PGL1 | 28 | 5.67 | 255 | 11 | 21 | 230 | 7.67↑ |
| 27      | gi|9238|46509 | Proteasome subunit alpha type-1-A | PAF1 | 30.4 | 5.09 | 277 | 8 | 18 | 326 | 2.39↑ |
| 28      | gi|3839|0459 | ATPase alpha subunit | ATPA | 55.3 | 5.14 | 507 | 23 | 29 | 588 | 3.15↑ |
found to be expressed only in the Bnclv-like background were detected in the wild type using qPCR, such as CPN20, mMDH1 and S6PDH (Figure S1). This may be due to a post-transcriptional modification of mRNAs. Collectively, at the protein level, three biological processes made major contributions to the abnormal development of the IM in Bnclv-like. The up-regulation of proteins in the metabolic processes and cytoskeleton formation could provide enough energy and faster transportation of cellular materials for fulfilling the higher activity of the Bnclv-like in IM. On the other hand, the down-regulation of proteins involved in ROS metabolism might have a positive influence on the maintenance of stem cell activity. In general, the qRT-PCR results showed that the transcriptional and protein levels of the fourteen proteins were the same.

Discussion

In present study, we obtained a natural mutant of rapeseed named as Bnclv-like, which exhibited abnormal inflorescence formation. We speculated that the Bnclv-like phenotype was caused by abnormal development of the IM. So, the proteomic analysis was implemented to further investigate the unusual IM development in Bnclv-like. Using the GO classification and KEGG pathway analysis of the differential proteins between the Bnclv-like mutant and wild-type IM, we found that these differential proteins were mainly involved in metabolic processes, responses to stimulus and cellular component organization or biogenesis.

Plants need a lot of ATP for energy during the whole growth and development process (Parker et al., 2006; Kang et al., 2012). From KEGG pathway analysis, we identified...
seven proteins belonging to the glycolysis/gluconeogenesis pathway and seven proteins participating in the process of carbon fixation in photosynthetic organisms. GAPC1, TPI, and TIM are involved in these two pathways simultaneously. GAPC1 (Phosphorylating glyceraldehyde-3-P dehydrogenase) is a highly conserved cytosolic enzyme, but it is also thought to be related to other cellular functions apart from its participation in glycolysis. The gapc1 mutant exhibits delayed growth, altered silique morphology, and decreased ATP level and respiratory rate (Rius et al., 2008). However, GAPC1 overexpression had no significant influence on seedlings in the vegetative stage, which presented a seed-specific expression pattern of GAPC1 (Guo et al., 2014). In plants, triose phosphate isomerase (TPI) participates in several metabolic processes, including gluconeogenesis, glycolysis, and the Calvin cycle. One or various TPIs are present in plant genomes and are located in the cytoplasm and chloroplast (cTPI and pdTPI), respectively. cTPI is involved in glycolysis, whereas the chloroplastic enzymes participate in the Calvin cycle (Turner et al., 1965; Kurzok and Feierabend, 1984; Tang et al., 2000; Chen and Thelen, 2010). In Arabidopsis, the lack of pdTPI results in termination of the transition from vegetative to reproductive stages or plants suffers from stunted growth and abnormal development of chloroplasts (Lopez-Castillo et al., 2016). In the present study, the expression of TIM and TPI was upregulated, which might contribute to energy metabolism in the IM in the Bnclv-like mutant.

Actin plays a key role in regulating organ growth, cell proliferation and floral bud morphogenesis from vegetative to reproductive stages in plants (Feng et al., 2006; Zhang et al., 2013; Zheng et al., 2013; Wu et al., 2016). The Arabidopsis ACT7 gene is expressed in rapidly developing tissues, in which the highest level of ACT7 mRNA could be detected in developing vegetative organs (McDowell et al., 1996). In addition, ACT7 is the only actin gene in Arabidopsis that responds strongly to auxin (McDowell et al., 1996). A recent study demonstrated that ACT7 participated in the process of TWISTED DWARF1 (TWD1) mediation of auxin transport. Although ACT7 may be an indirect-TWD1 interactor, it controls the presence of efflux transporters at the plasma membrane. As a consequence, act7 and twd1 mutants shared developmental and physiological phenotypes indicative of defects in auxin transport (Zhu et al., 2016). Our data showed that the expression level of ACT7 protein was significantly up-regulated in the Bnclv-like mutant. Taken together, the highly expressed ACT7 in Bnclv-like mutant might promote cell division and growth during IM development.
ROS are well-known stress responding molecules in plants and animals which can be increased dramatically in response to pathogens and environmental stresses (Finkel and Holbrook, 2000; Swanson and Gilroy, 2010). A recent study indicated that redox participated in the regulation of plant stem cell fate (Zeng et al., 2017). O$_2^-$, the precursor for most ROS, can be transformed into H$_2$O$_2$ by superoxide dismutase (SODs). Ideal concentrations of O$_2^-$ can stabilize the activity of stem cells, but excess H$_2$O$_2$ can suppress or even disrupt their activity (Zeng et al., 2017). Two SODs, SOD2, and FSD1, were altered significantly in the Bnclv-like IM. In a previous report, these two proteins were found to be strongly expressed in the differentiating peripheral zone instead of the stem cells as a result of the different distribution of O$_2^-$ (Yadav et al., 2014; Zeng et al., 2017). In the present study, the expression of SOD2 was up-regulated dramatically, which could catalyze the transformation from O$_2^-$ to H$_2$O$_2$ to suppress stem cell activity. However, FSD1 showed a more significant down-regulation than SOD2, which may compensate for the elevated activity of SOD2. Another study indicated that ROS were crucial molecules in triggering meiotic fate acquisition in maize (Kelliher and Walbot, 2012), which demonstrated an important role of ROS in cell fate determination.

The PPI network showed that proteins involved in cell metabolism, cytoskeleton formation and ROS metabolism interact with each other. Due to their crucial role in cytoskeleton formation and ROS metabolism in cell development, ACT7 and FSD1 were selected for further analysis. The number of proteins interacting with ACT7 and FSD1 accounted for $>70\%$ in all interacting with proteins, indicating the vital role of these two processes in regulating the development of the Bnclv-like mutant IM.

Among the proteins interacting with ACT7, PRF1 encodes profilin. The *in vitro* studies had shown that the profilin-actin complexes were associated with the barbed ends of actin filaments and promoted actin polymerization by reducing the critical concentration and increasing nucleotide exchange on G-actin (Pollard and Cooper, 1984; Pantaloni and Carlier, 1993). In *Arabidopsis thaliana*, PRF1 participates in stochastic actin dynamics by regulating formin-mediated actin nucleation and filament elongation in the process of axonal cell expansion (Cao et al., 2016). Consistent with our results, the expression of PRF1 in the Bnclv-like mutant is up-regulated relative to the wild type, together with ACT7, which is consistent with the enrichment of ACT7. Fructose 1, 6-biphosphate aldolase (FBA) in plants is a key metabolic enzyme in glycolysis and gluconeogenesis in the cytoplasm (Gross et al., 1999). FBA8 is a member of the cytoplasmic fructose 1, 6-biphosphate aldolase family. A recent study showed that the knockout of the *FBA8* gene resulted in slight alternations of the actin cytoskeleton morphology of guard cell and reduced the rate of stomatal closure in cope with decreased humidity (Garagounis et al., 2017). Moreover, the *fba8* mutant displayed sterility (Lu et al., 2012). *In vitro* experiments confirmed the interaction between FBA8 and actin in *Arabidopsis* (Lu et al., 2012). Due to the significant role in cytoskeleton formation and glucose metabolism, FBA8 may provide a link between these two processes. The up-regulation of PRF1 and FBA8 could enhance the development of IM through their interaction with ACT7.

Among the proteins interacting with FSD1, *Arabidopsis* chloroplast CHAPERONIN 20 (CPN20) can form tetramers *in vitro*, which is a cofactor of chaperonin (Koumoto et al., 1999). In *Arabidopsis*, CPN20 is speculated to have many functions in the chloroplast independent of its co-chaperonin, such as regulating abscisic acid signaling transduction and mediating iron SOD activity (Kuo et al., 2013; Zhang et al., 2014). CPN20 was identified as a mediator for activating FeSOD by direct interaction *in vivo* and *in vitro* (Kuo et al., 2013). mMDH1 encodes a mitochondrial malate dehydrogenase, which participates in the transformation of malic acid and oxaloacetic acid in the tricarboxylic acid cycle. A decreased activity of mMDH1 has a up-regulated influence on photosynthetic metabolism, which leads to smaller rosettes and decreased fresh weight (Linden et al., 2016; Sew et al., 2016). The mmdh1mmdh2 double mutant plants exhibit a significantly higher rate of leaf respiration, low net CO$_2$ assimilation, limitation in photosynthetic rate, and slow-growth phenotypes in rosettes (Tomaz et al., 2010; Linden et al., 2016). In the Bnclv-like mutant, the upregulation of CPN20 and mMDH1 contribute to the protein biosynthesis and biomass accumulation to maintain the accelerated activity of IM. Besides, the interaction among CPN20, mMDH1, and FSD1 could represent the transformation from energy metabolism to reactive oxygen metabolism in the plant body. A further study should be undertaken to reveal the relationship between these two processes.

We found that the three proteins, TPI, GAPC1, and ACHT1, showed interactions between with ACT7 and FSD1. The first two of them participate in glycometabolism, while ACHT1 is involved in regulating photosynthetic electron transport progress (Dangoor et al., 2012). Therefore, we proposed that energy metabolism could be a link connecting cell organization and superoxide metabolism. Taken together, in protein level, three biological processes showed a great contribution to the abnormal development of Bnclv-like mutant and the understanding of interaction between these proteins could be key to uncover the inner mechanism of IM development. This study provided clues for the further study of the Bnclv-like mutant in *B. napus* and the mutant was also a useful material for the study of IM development in *B. napus*.

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Conflicts of Interest
The authors declare no conflict of interests.

Author Contributions
KZ, WZ, RS and SX performed most of the experiments and wrote the manuscript. KL, YY and YL helped perform the phenotype analysis. ZW, JC and YL participated in data analysis. XT conceived the study and revised the manuscript.

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Supplementary material

The following online material is available for this article:
Figure S1 - The relative expression levels of selected genes which changed significantly in the 2-DE result.
Table S1 - Primers used for qRT-PCR.

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