Profiling of indole metabolic pathway in thermo-sensitive Bainong male sterile line in wheat (*Triticum aestivum* L.)

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Received: 7 September 2018 / Revised: 17 October 2018 / Accepted: 12 November 2018 / Published online: 28 November 2018
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Abstract Bainong male sterile (BNS) wheat (*Triticum aestivum* L.) is a thermo-sensitive genic male sterile line with excellent sterility and self-restoration. We focused on transcriptional profiles of differentially expressed probes between BNS sterile and fertile anthers. Anthers, rachis and spikes from sterile line and fertile line were collected. Extracted RNA was assayed using wheat expression microarray and Gene Ontology was analyzed using Cytoscape with ClueGO. An indole (indole-3-acetic acid: IAA) metabolism pathway sub-network was almost formed in all differentially expressed profiles between sterile and fertile samples. IAA sub-network contained four nodes of indole and alkaloid metabolism connecting main network via indole compounds. This sub-network was absent in rachis and intact in transformed fertile anthers, which was the main differently expressed metabolism pathway in F₁ anthers with restorer genes. Alkaloid metabolism was absent in sterile anthers. Abnormal metabolism of IAA may be involved in BNS sterility. BNS transformation may be regulated by the production of IAA and alkaloid metabolism pathway, which favor the safe utilization of the sterile line in hybrid wheat production.

Keywords Alkaloid metabolism · BNS male sterility · Gene expression microarray · IAA metabolism · Wheat

Introduction

Plant male sterile lines are the core materials for the utilization of heterosis. There are three main types of male sterile lines that can be used for wheat breeding and hybrid seed production. The first is three-line system, originated from cytoplasm-nucleus interaction male sterility (CMS). The second is two-line system, from photo-thermo-sensitive male sterility (PTGMS), and the third is chemical hybrid male sterility (CHM) treated with chemical androgenicids (Murai et al. 2008). Among them, the two-line system is the most valuable line for hybrid wheat production. It is a simplified system, with rich genetic background, and plump seeds of F₁ (Murai et al. 2008; Singh et al. 2015; Zhao 2010).

A series of two-line system male sterile lines were found and applied in recent 20 years in China (Li et al. 2006, 2009, 2011a; Zhang et al. 2006; Zhao 2010). BNS is a thermo-sensitive genic male sterile line with good sterility and good self-conversion. Its F₁ displays strong heterosis when it crosses with special father varieties (Fan et al. 2015; Li et al. 2009; Ma et al. 2013; Ning et al. 2011; Zhang et al. 2013a; Zhou et al. 2010). The characteristics of sterility, transformation and restoration of BNS have been observed systematically, and the genetic mechanisms have also been preliminarily studied (Fan et al. 2015; Ma et al. 2013; Ning et al. 2011; Sun et al. 2016; Zhang et al. 2013a), but its physiological mechanisms are seldom reported.

The occurrence and physiological mechanism of PTGMS were reported frequently in crops such as rice and rapeseed. It is generally believed that environmental factors such as light

Electronic supplementary material The online version of this article (<https://doi.org/10.1007/s12298-018-0626-0>) contains supplementary material, which is available to authorized users.

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and temperature induced sterile gene expression at a certain stage of anther development, which changed the metabolism pathway and led to pollen abortion (Pan et al. 2014; Ulmasov et al. 1997; Zhao et al. 1996). In this process, plant hormones are considered to be the important mediators. The content levels of Indole-3-acetic acid (IAA), gibberellic acid (GA₃), abscisic acid (ABA), and zeatin riboside (ZR) and so on, especially IAA, showed significant changed in a variety of plant male sterile lines. For example, rice Nongken 58S is a photo-thermo-sensitive male sterile line, its photo-sensitive organ was leaf, and IAA mediated the information from leaf to anther (Li et al. 2000). The recessive sterility site (ms33) of *Arabidopsis thaliana* could induce male sterility, accompanied with reducing indole-3-butyric acid and endogenous GA₃ (Fei et al. 2004). The concentration of endogenous IAA, IBA and GA₃ in two-line hybrid wheat C49S decreased significantly during the development of young ear (Zhang et al. 2001). The content of IAA in leaves and anthers of sterile BNS also significantly decreased when it differentiated from pistil to trinucleus (Zhang et al. 2016, 2013b). It suggests that the occurrence of male sterility in plant may be closely related to indole metabolism.

One of the risks of the utilization of the two-line hybrid wheat is that abnormal meteorological conditions for fertility transformation may lead to the increase of self-fertilization rate (Du et al. 2016; Qin et al. 2018) and would reduce the purity of hybrid wheat seeds, which could cause a fatal event for hybrid wheat production. For safely using of two-line male sterile system, it is necessary to control fertility conversion by artificial means with physiology mechanism and more research on metabolism in male sterility is indispensable.

Transcription microarray is a high throughput tool for differently expressed genes filtering and analysis (Daraselia et al. 2007; Hu et al. 2016; Meng et al. 2017). We used expression profile database and protein profile database to analyze metabolic pathway changes directly. In order to study the physiological mechanism of BNS sterility, anthers and rachis tissue were collected from typical sterile plants and fertile plants. Differentially expressed genes between different groups were detected by Affymetrix microarray, and metabolic pathways involved in the differentially expressed genes were analyzed. Our study may benefit the mechanism understanding and applying of sterility of BNS in wheat breeding.

Materials and methods

Sterile line materials

BNS line is an original male sterile line in our laboratory. It is planted and preserved by bagged every year in Huixian

wheat breeding base of Henan province (China). BNS wheat develops into male sterility when it is sowed from October 1st to 10th in local area. The percentage of fertile pollen gradually increases after October 10th. The self-setting rate of the line reaches the highest when it is sowed around November 10th (Du et al. 2016; Qin et al. 2018). The BNS in our experiment was sown from October 1st to November 17th once every 8 days and 7 times in total. In order to compare the difference between temperature restoring fertility by BNS itself and restoring fertility by restoring gene, a combination of “BNS × Chinese Spring” was set up last year. Chinese Spring is a high restorer line of BNS. F₁ of “BNS × Chinese Spring” was normally fertile even it was sowed in October 9th which is the typical sterile sowing date of BNS, and also is the local wheat sowing date. The planting pattern in the field was 3 m for row length and 0.23 m for row width, and the distance between two plants was 0.13 m. Two rows were planted and were separated by one empty row, which provided big space for plants growth and development.

Sampling

Sterile samples were collected from BNS sowed on 1st October, and fertile samples were collected from BNS sowed on 18th November, and F₁ samples were collected from F₁sowed on 9th October. The sampling time was determined according to the stage of panicle differentiation. The number, sampling date, and panicle differentiation stage of all samples were shown in Table 1. The sampling method was as following: the ears were collected at 7 am in field, and then they were taken to laboratory to be checked the microspore development stage under general microscope. The anthers of normally developed stage were peeled from spikes on ice bag, and were collected as anther samples. Then they were weighted, frozen for 1 min in liquid nitrogen and transferred to − 72° for storage. The ears whose spikes were removed were rachis samples which were weighed, frozen and preserved.

Microarray hybridization and analysis

Total RNA was extracted using Trizol method according to the manufacturer instruction (Invitrogen, USA). Each tissue sample of 100 mg was grinded in mortar with liquid nitrogen and 1 ml Trizol reagent was added. Equal volume chloroform was added and centrifuged at 2700 g 4 °C for 20 min. Supernatant was transferred to an equal volume isopropanol tube and oscillating mixed followed by stewing for 10 min at room temperature. After centrifuging at 2700 g 4 °C for 20 min, 70% ethanol was added into the treated RNA and centrifuged at 2700 g for 5 min. Dried RNA was dissolved by 32 µl DEPC water (Diethy

Table 1 Details of 9 samples

Samples	Sowing date	Development stage and organ	Sampling date
Spike1	9th October	Spike in anther differentiation stage	20th March
Ste-anther	9th October	Anther of tetrad stage, sterile plant	1st April
Ste-rachis	9th October	Rachis of tetrad stage, sterile plant	1st April
Fer-anther	17th November	Anther of tetrad stage, fertile plant	10th April
Fer-rachis	17th November	Rachis of tetrad stage, fertile plant	10th April
F1-anther	9th October	Anther of tetrad stage, F ₁ plant	1st April
F1-rachis	9th October	Rachis of tetrad stage, F ₁ plant	1st April
Ste-mono	9th October	Anther of mononucleate stage, sterile plant	3th April
Fer-mono	17th November	Anther of mononucleate stage, fertile plant	12th April

Spike1 spike in anther differentiation stage, *Ste-anther* anther of tetrad stage, sterile plant, *Ste-rachis* rachis of tetrad stage, sterile plant, *Fer-anther* anther of tetrad stage, fertile plant, *Fer-rachis* rachis of tetrad stage, fertile plant, *F1-anther* anther of tetrad stage, F₁ plant, *F1-rachis* rachis of tetrad stage, F₁ plant, *Ste-mono* anther of mononucleate stage, sterile plant, *Fer-mono* anther of mononucleate stage, fertile plant

pyrocarbonate, Promega, USA). RNA concentration and integrity were assayed using spectrometer NanoDrop (Thermo Scientific, USA) and Agilent 2100 bioanalyser (Agilent, USA).

Affymetrix Wheat Genome Array was designed based on the database of GeneBank and dbEST which contained 61127 probe sets representing 55052 transcripts (<http://www.affymetrix.com/estore/>). Wheat RNA samples were labeled, hybridized (Shanghai Yeslab Biotechnology, China) and scanned using the GeneChip Scanner 3000 (Affymetrix, Santa Clara, CA USA) according to the manufactures instruction. Array images were scanned and analyzed by Affymetrix Microarray Suite (MAS, version 5.0) and signal intensity of probes were normalized using Robust Multi-array normalization algorithm method (RMA, GeneSpring GX v11.5, Agilent). The cut-off of false discovery rate (FDR) was set as 0.05. The log₂ Ratio of differently expressed genes were analyzed by method of paired error weighted mean and significance cut off value was $\log_2(\text{Ratio}) > 1$ (value ≥ 2).

Validation of differently expressed genes by qRT-PCR

Validation of differently expressed genes was performed using qRT-PCR method (Crismani et al. 2006). Genes of *ATP1*, *Hsp23.5*, Aldehyde dehydrogenase *ALDH2* and malate dehydrogenase *CMDH* were selected for expression analysis because they differently expressed both in previous proteome research (Li et al. 2011b, 2015; Su et al. 2013) and present microarray results. The primers of 4 detected genes were: *atp1* primer F TTCCGCGA-TAATGGAATGCACGCA, R AGCTACCTGCACCTGTCTGGTCCG; *hsp23.5* primer F GCACCTCGA TGCGGCGGTTGTACT, R AGGACGACGACGC GGTGTACCTCA; *cmdh* primer: F TGGTGCAGAC

CAGCCTGTTATTCT, R CCGCAACATTCA-CACCAGTGCAGGCC; *aldh2* primer: F TTTCACCAAC AACCTGGACACGGC, R CTCCTACCGATGCCGCT-CATCTT. Samples of Ste-anther, Ste-rachis, F1-anther, F1-rachis, Ste-mono, Fer-mono were performed by qRT-PCR with a housekeeping genes glyceraldehyde phosphate dehydrogenase (GAPDH) as reference gene on a real time PCR instrument (Roche LightCycler 480 PCR) (Li et al. 2015). The primers of GAPDH were F CCCTCA-GACTCCTCCTTGATAGC, R TGACTGCAACCCAGA AGACTGTT.

Metabolism analysis

There were 9 samples in total. Sample Spike1 was young ears with un-differentiated floret. Florets had differentiated in other 8 samples. These 8 samples consisted of 4 pairing materials. Comparisons of each pair could imply different meanings. Ste-anther and Ste-rachis indicated anther and non-anther tissue of sterile BNS. Fer-anther and Fer-rachis indicated anther and non-anther tissue of fertile converted by BNS itself. F1-anther and F1-rachis originated from the F₁ of “BNS × China Spring”. The plant development of F₁ was also fertile under the condition of sterility, therefore, the difference between F1-anther and F1-rachis was that between anther and non-anther tissues with dominant restore gene. Ste-mono and Fer-mono indicated sterile and fertile anther tissue that developed at the stage of mononucleate. More information could be acquired from these materials based on more samples comparisons. For examples, more genes and metabolic differentially expressed information could be obtained when the undifferentiated floret Spike1 was compared with the anther, rachis and other samples.

Differently expressed genes (DEGs) in each paired samples were obtained according to the cut off value ≥ 2

based on normalized intensities of each probe (fold change = $2^{(\text{normalized intensity 1} - \text{normalized intensity 2})}$). GO (Gene Ontology) and pathway analysis were performed using Cytoscape 3.5.1 platform with a plug of ClueGO 2.3.1 (Bindea et al. 2009), (<http://www.ici.upmc.fr/cluego/cluegoDescription.shtml>). Affymetrix probe IDs were inputted into the ClueGO software and searched against wheat database. GO analysis was focused on the biological process and network of metabolism were analyzed on differently expressed genes (Shannon et al. 2003).

Results

Microarray analysis and hybridization quality evaluation

GeneSpring GX software (Agilent, USA) was employed to normalize the microarray results using RMA method by loading the CEL files of array. Valid probes of 15720 were acquired by RMA randomized method. Quantitative PCR results showed that the expression tendency of four genes Hsp23.5, ALDH, CMDH and ATP1 were highly consistent with microarray, and the expression level ($\Delta\Delta\text{Ct}$) of four genes in above two methods was also close (Fig. 1). The coincident results implied that the microarray results were reliable.

Metabolism analysis of differently expressed probes

The expression profiling of 15732 probes between the paired samples, different tissues (anther and rachis), and different developmental stages (early and late anther) were analyzed. The details of up and down regulated probes between paired

samples were summarized in Table 2. The chip probe set signal was normalized, followed by calculating with formula of $2^{-(\text{normalized expression level 1} - \text{normalized expression level 2})}$, and the count of up-regulated probes was approximately equal to the down-regulated probes. In Table 2, only the counts of up-regulated or down-regulated probes involved in IAA pathway were showed.

Differently expressed genes were input into the software ClueGO, and biological process analysis was performed with a set of two tails test, 4–5 genes involved and kappa score = 0.4. Metabolism pathway was analyzed. In some matched groups, cutoff value was increased so the counts of differently expressed genes decreased to less than 1000. Gene probes were decreased to about 200 when the key genes were analyzed.

The results indicated that there existed a stable and relatively independent sub-network in the main network of differently expressed genes between anthers and other tissues as well as other sterility and fertile samples (Fig. 2a–c). This metabolic sub-network consisted of four nodes, corresponding to 4 GO terms, which were indole biological synthesis, indole metabolism, alkaloids biosynthesis and alkaloids metabolism process (Fig. 2d). The sub-network was connected to the main network via GO nodes containing indole-containing compounds (Fig. 2a–c). As the function of the nodes in sub-network was mainly indole biosynthesis and metabolism, it was called as indole pathway sub-network (IAA metabolic sub-network).

The universality and stability of the sub-network were also represented by the following two facts. The main network changed into a few simple sub-networks in which IAA sub-network still existed and kept completely when cut-off value was set more strictly such as increasing fold-change or enlarge kappa score of ClueGO (Fig. 3). Sixteen

Fig. 1 Relative expression level ($\Delta\Delta\text{Ct}$) of four genes using microarray and qRT-PCR assay. Bar graph represented the relative expression of four genes. Up regulated genes were displayed above the X axis and down regulated genes were displayed down the X axis

Table 2 Differently expressed genes over two fold-change between paired samples involved in IAA metabolism

Sample comparing	Differently expressed probes	Down/up
Spike1 vs Ste-rachis	1696	Up
Ste-anther vs Ste-rachis	1938	Up
Ste-anther vs Fer-rachis	2279	Up
Fer-anther vs Fer-rachis	3096	Up
Ste-anther vs F1-anther	1468	Down
Ste-anther vs F1-rachis	2133	Up
Fer-anther vs F1-rachis	2720	Up
F1-anther vs F1-rachis	2421	Up
Spike1 vs Ste-mono	3507	Down
Fer-anther vs Ste-mono	667	Up
F1-anther vs Ste-mono	1959	Up
Spike vs Fer-mono	2985	Down
Ste-anther vs Fer-mono	1339	Up
F1-anther vs Fer-mono	1065	Up
Ste-mono vs Fer-mono	967	Down

IAA metabolic pathway was detected in these probe profilings. But IAA metabolic pathway was not found in down-regulated probe profilings between Spike1 versus Ste-rachis which was not shown in the table

meaningful comparisons among 9 samples were analyzed, in which the sub-network was found in 14 pairs of comparisons (Table 2).

It was reported that the deficiency of IAA was one of the important physiological manifestations of crop male sterility. Here, the abnormal metabolic pathway of IAA was found in the metabolic pathway of differential expression, which meant that this action also could occur in BNS. By the analysis of the metabolic pathways of 14 differentially expressed probe profiles, some essential characteristics of the sub-metabolic network were described as following.

1. Table 2 showed the possible meaningful comparisons among the nine samples. It could be found from the table that IAA metabolic sub-network existed in fertile anthers comparing with rachises, such as Ste-anther versus Ste-rachis, Ste-anther versus Fer-rachis, Ste-anther versus F1-rachis, which formed only in up-regulated probes. When sterile anthers are compared with fertile anthers, the sub-metabolic pathways appeared in down-regulated probes of fertile anthers. For example, the sub-metabolic pathways appeared in F1-anther and Fer-mono compared with Ste-anther versus Fer-mono, Ste-anther versus F1-anther, and Ste-mono versus Fer-mono. The sub-network appeared in the up-regulated probe of F₁ fertile anther such as F1-anther versus Ste-mono, F1-anther versus Fer-mono, F1-anther, when F₁ fertile anther was compared with BNS fertile anther restored by BNS itself.
2. Indole metabolism pathway was the main metabolism of different expressed genes in typical sterile and fertile

material pairs. Figure 3 displayed a ClueGO network mapping 179 differentially expressed probes (four fold change) which came from comparison between Ste-mono (sterile anthers at mononucleate stage), and Fer-mono (fertile anthers at mononucleate stage). There were only two sub-networks with more than one node in the enriched metabolic map, one of which was indole pathway. This characteristic was also found in up-regulated 264 probes over eight fold change in F1-anther versus F1-rachis, and up-regulated 213 genes of three fold change in Fer-anther versus Ste-mono. Figure 4a showed that the indole metabolic process accounted for a large proportion in up-regulated probes between anthers of tetrad stage in sterile plant, and panicle of tetrad stage in sterile plant. It was also found in the down-regulated probes between sterile monocyte anthers and fertile monocyte anthers (Fig. 4).

3. Indole metabolic pathway was not completely lost in sterile anthers, but it presented incomplete or low expression level. Figure 5 showed a metabolic diagram of down regulated profiling between Spike1 (sterile young ear) and versus Ste-mono (sterile mononucleate anthers). There were only 2 IAA nodes, and no nodes of alkaloid biosynthesis and alkaloid metabolism in the IAA metabolite sub-network. The complete IAA metabolic pathway was formed, which indicated that IAA metabolism down regulated in sterile anthers when sterile anthers were compared with fertile anthers (Fig. 2a–c).
4. Indole metabolism sub-network was a terminal metabolism pathway which located in the same position outside of main network in all involved network. It

Fig. 2 Network diagram of differentially expressed probe profiling. The position of the IAA metabolic sub-network was displayed with black arrow. **a** Network diagram based on 1331 up-regulated probes over three fold-change between fertile monocyte anthers (Fer-anther) and F1 tetrad panicles (F1-rachis), which showed the complex metabolic network. **b** Network diagram based on 875 up-regulated probes over three fold-change between anthers at tetrad stage of sterile plant (Ste-anther) and anthers at F1 tetrad stage (Ste-rachis), which showed

indoxal compound was the connection node from the sub-network to the amino acid metabolism main network. **c** The main network diagram of 347 up-regulated probes over five fold-change between anthers at tetrad stage of sterile plant (Ste-anther) and anthers at F1 tetrad stage (Ste-rachis), which showed IAA sub-network and regulation route. **d** The structure of IAA metabolic sub-network. It contained 4 nodes of indoxal biosynthesis, indoxal metabolism, alkaloid biosynthesis and alkaloid metabolism

linked to the main network of amino acid, organic acids and monoterpene metabolism nodes by a node containing indole compound (Figs. 2a–c, 3). This

metabolism was a relatively stable and independent metabolic pathway, and inter-regulated with amino acid and monoterpene metabolism. We termed indole

Fig. 3 The Network analysis of 179 down-regulated over four fold-change probes between sterile mononucleate anthers and fertile mononucleate anthers. The main network disappeared, and only 2 sub-networks of indole metabolism and coenzyme A biosynthesis metabolism were kept

metabolism as a terminal metabolic sub-network for its position and independence.

5. Indole metabolism sub-network interacted with its main network. Figure 2c was a main network with mapping of 347 up-regulated probes which were over five fold-change between anthers and panicles at tetrad stage of sterile plant. It was showed that the pathway of information flow between the sub-network and its main network metabolism was as following: from main network to a GO node containing indoxyl, to IAA metabolism, to alkaloids metabolism; then back to IAA metabolism to the node of GO containing indoxyl, finally, returned to the main network. The yellow nodes were incoming nodes, and the green were outgoing nodes.

Annotation of key genes involved in IAA metabolite sub-network

The consistency and stability of indole metabolic sub-network in fertile anthers indicated that IAA metabolism would play an important role in the development of anthers and pollen in wheat. In order to further analyze the metabolic mechanism of the sub-network, “Associated Genes Found” was extracted from the results of ClueGo analysis (Table 3). There were 4 probe genes in total in structure GO nodes, they were Ta.2863.1, Ta.15150.1, Ta.15151.1 and LOC778393. The count of probe genes in two connecting GO nodes were more than that in structure GO nodes. Their counts were determined by the size of the main network. The larger the main network was, the more the connection between IAA sub-networks and main

network would be. All of the non-repeat probe genes in 2 connecting GO nodes of IAA metabolic network over three fold-change are shown in Table 3, which contained 17 and 12 genes respectively. The function of these probe genes were annotated from Affymetrix probe database (<http://www.affymetrix.com/analysis-netaffy/>) in Table 4. From the table, it can be seen that protein LOC778393 shared by six GO terms was a male sterile protein of *Arabidopsis thaliana*. The probe gene Ta.2863.1 was similar to 41KD heat shock protein Dan J in *Arabidopsis thaliana*. Among these genes in connecting GO nodes, Wcat1 (Ta.442.1) was a peroxidase active protein, Ta.27477.1 was a oxalate oxidase GF-2.8 precursor, the rest were or EST locus, or putative protein locus with unknown function.

Discussion

Plant auxin hormones contain indole molecules including indole-3-acetic acid, acid-indoleacetic and heteroauxin which are indispensable substances for plant growth, organ building, nutrient transport and development. It has long been found that the contents of IAA decreased in male sterile young panicles of plants, such as rice (Li et al. 2000; Zhao et al. 1996), maize (Tang et al. 2003) and *Arabidopsis thaliana* (Fei et al. 2004), which led to imbalance of hormone ratio, like high temperature and water stress, then reduced the ability of transporting nutrients and induced male sterility occurrence. IAA deficiency was also found in PTGMS. In rice, Nongken 58S, IAA concentration in leaves of sterile line was only 1/3 of that in restored line (Li et al.

a **b**

Fig. 4 Clustering analysis of IAA metabolism of typical differential probe profiles by enrichment. Each process item proportion was displayed with different color in pie graph. **a** The cluster diagram of 347 up-regulated probes over five fold-change between anthers of

tetrad stage in sterile plant (Ste-anther), and panicles of tetrad stage in sterile plant (Ste-rachis). **b** The cluster diagram of 481 down-regulated probes over threefold-change between sterile monocyte anthers (Ste-mono) and fertile monocyte anthers (Fer-mono)

2000; Pan et al. 2014). IAA content in anthers of sterile line was about 80% of fertile line in wheat male sterile line C49S and BNS (Zhang et al. 2006, 2013b, 2016). Based on the transcriptional profiles, we found that IAA metabolism

was a typical differential metabolic pathway in network between anthers and young panicles tissues. These results were consistent with previous studies on BNS physiological detection (Zhang et al. 2013b), which suggested that the

GO ID	Node name	Associated genes found
GO:0042431	Indole biosynthetic process	LOC778393, Ta.2863.1, Ta.15150.1, Ta.15151.1
GO:0042432	Indole metabolic process	LOC778393, Ta.2863.1, Ta.15150.1, Ta.15151.1
GO:0009820	Alkaloid metabolic process	LOC778393, Ta.2863.1, Ta.15150.1, Ta.15151.1
GO:0009821	Alkaloid biosynthetic process	LOC778393, Ta.2863.1, Ta.15150.1, Ta.15151.1,
GO:0042430	Indole-containing compound metabolic process	LOC778393, Ta.2863.1, Ta.15150.1, Ta.15151.1, Ta.1118.1, Ta.27477.1, Ta.3840.3, Ta.4225.1, Ta.431.2, TaAffx.12340.1, TaBx1-A, Ta.14944.1, wcat1, Ta.6643.3, Ta.19271.3, Ta.25865.1, Ta.25867.1
GO:0042435	Indole-containing compound biosynthetic process	LOC778393, Ta.2863.1, Ta.15150.1, Ta.15151.1, Ta.1118.1, Ta.27477.1, Ta.3840.3, Ta.4225.1, Ta.431.2, TaAffx.12340.1, TaBx1-A, Ta.14944.1

Table 4 Annotation of important probe genes related to BNS male-sterility

Probe ID	gb ID	Annotation
Ta.11286.1	BQ169778	Male sterility protein-like (LOC780568) in <i>Arabidopsis thaliana</i>
Ta.2863.1	CK210585	Putative DnaJ protein(heat shock protein 41KD) in <i>Arabidopsis thaliana</i>
Ta.15150.1	CD878742	Wheat putative protein-coding gene
Ta.15151.1	CA691622	Wheat transcribed locus in <i>Triticum aestivum</i>
Ta.1118.1	BQ168573	A protein in <i>Arabidopsis thaliana</i>
Ta.3038.1	CK157008	Putative protein in <i>Arabidopsis thaliana</i>
Ta.27477.1	CD373670	Oxalate oxidase GF-2.8 precursor in <i>Triticum aestivum</i>
Ta.3840.3	CA608029	AKIN beta1 (<i>Arabidopsis thaliana</i>)
Ta.4225.1	BJ276566	Expressed protein in <i>Arabidopsis thaliana</i>
Ta.431.2	BJ296777	A protein in <i>Arabidopsis thaliana</i>
Ta.14944.1	CA685324	Transcribed sequence in <i>Arabidopsis thaliana</i>
Wcat1(Ta.442.1)	D86327.1	Wheat oxidative stress, catalase activity

metabolism of IAA and alkaloid metabolism are involved in the process of BNS sterility occurrence. The abnormality of IAA metabolism was one of the main physiological characteristics, which played an important role in physiology and sterility regulation of sterility.

IAA metabolism closely related to the occurrence of BNS sterility. The metabolism of IAA was formed in different expressed genes between sterile and fertile anthers, as well as anthers and other tissues. This pathway was found incomplete without alkaloid in sterile anthers. IAA pathway was the main metabolic pathway of differently expressed genes in fertile anther of F_1 plant with restoring genes. This metabolism pathway was still formed if we adjusted the fold-change to 8, which implied that the metabolism of IAA was an important metabolic pathway for the normally development of wheat anthers. And those of weakening, incomplete and missing metabolisms will directly affect anther development. In BNS, the metabolism of IAA was abnormal and defective, which may lead to anther dysplasia and then pollen abortion. The information regulation route between the metabolism of IAA and the metabolism of main network is as following: from main network to IAA, from IAA to alkaloid, from alkaloid to IAA, and then back to the main network. This regulation route not only indicated the importance of IAA metabolism, but also implied that alkaloid metabolism was an important component of IAA metabolic pathway. But the relationship between alkaloid metabolism and wheat male sterility is not lucid and has rarely been reported in the past. Therefore, further study is need to investigate the mechanism of how male sterility genes affect alkaloid metabolism, and how alkaloid metabolism affects pollen development, and the relationship between IAA and the alkaloid as well as how they regulate sterile process.

IAA metabolic pathway was a terminal metabolic pathway. In fertile anthers, all IAA metabolic sub-network

kept the same structure, relatively independent, and located outside of the main network, and connected to main network by a node containing IAA compound. It was suggested that IAA metabolism was the terminal metabolism with product formation, and could form a phenotypic trait. Abnormal metabolism may lead to phenotypic abnormalities and change sterility phenotype. A lot of researches have been reported on this metabolism in the past. A decrease in IAA content was detected in almost all plant male sterile individuals (Fei et al. 2004; Li et al. 2000; Pan et al. 2014; Tang et al. 2003; Zhang et al. 2006, 2013b; Zhao et al. 1996). Using IAA or IAA synthesis inhibitors or similar chemical reagents to treat young spikes of male sterility in rice, wheat, maize and other crops could change their sterility. He and Xiao (1992) reported that the spray of IAA at first terminal stage of branch differentiation in rice Nongken 58S could significantly increase the fertility of anthers, and treating with IAA oxidase inhibitor could reduced the sterility. Zhang et al. (2001) found that the fertile anthers and self-seeding rate was increased 20% in thermo-photo-sensitive wheat male sterile C50S when it was treated with $50 \mu\text{g ml}^{-1}$ IAA. And Tang et al. (1996) induced rice male sterility and found that the IAA and other hormone in anther were significantly decreased when it was treated with chemical anthericide TO3. Kovaleva et al. (2018) reported that ABA and IAA control microsporogenesis in *Petunia hybrida* L. These characteristics of IAA pathway not only indicated that their metabolism was an important physiological mechanism of sterility, but also implied that IAA or analogues could effectively regulate sterility occurrence or transformation.

The expression profiling of BNS sterility gene was anther specific and constitutive expression. The differential metabolism pathway between sterile plant and fertile plant in BNS was the result of differential expression of sterile genes. Based on the microarray analysis results, the genes

related to the metabolic pathway were expressed in anthers but not in rachis, which indicated that these genes were specifically expressed in anthers. When we compared sterile anthers and fertile anthers, the complete metabolic pathway of IAA was formed, which indicated that the related genes were expressed in fertile anthers. When we compared sterile anthers and spike axes, IAA metabolic pathway partially formed which indicated that the sterile genes were also expressed in sterile anthers. Both results suggested that the gene expression of this pathway should be constitutive in anthers. These two characteristics of sterile gene expression, which have been found in our previous studies (Su et al. 2013; Zhao et al. 2014), provided two important clues to understand the mechanism of BNS sterility. One was that the temperature sensitive organ of BNS should be young ears and differentiated anthers, not rachis. The second was that the BNS sterile genes should be the functional mutation of key structure genes of pollen development, not the regulation genes.

Four key genes of male sterility were found. Among the four co-expressed genes in structural GO nodes and connecting GO nodes, two of which were annotated. One was Ta.11286.1 that codes Arabidopsis male sterile protein T16L24.80, and the other was Ta.2863.1, which is the gene of 41KD heat shock protein Dan J in *Arabidopsis thaliana*. Among the genes in connecting GO nodes, wheat peroxidase gene and oxalate oxidase precursor gene were annotated. For other genes, their function was unknown. Although the total number of annotated genes was relatively less, the annotated information still provided a few important clues for the probes of BNS male sterility mechanism. The male sterility protein LOC780568 was associated with sterility. The heat shock protein, a kind of temperature sensitive protein, was consistent with the characteristic of temperature-sensitive in BNS. There were a lot of reports on the relationship between heat shock protein and photo-thermo-sensitive male sterility (Al-Wahaibi 2011). HSP23.5 gene of the small heat shock protein was significantly down-regulation in BNS sterile plant (Li et al. 2011b; Su et al. 2013; Zhao et al. 2014), indicating that the transcriptome results were consistent with the proteome results. Wheat 17.9KD and 23.6KD small heat shock protein genes were also differently expressed in barley male sterile spikelets induced by high temperature (Oshino et al. 2007); For annotated genes in connecting nodes, peroxidase had detoxification effect (Feki et al. 2015), and oxalate oxidase activity was related to apoptosis (Simmonds et al. 2004), which were closely related to the occurrence and recovery of male sterility. The analysis of male sterility and fertile transcriptional profiling of cotton (Suzuki et al. 2013) and rape (Zhou et al. 2017) showed that the indole-3-acetic acid amino synthetase metabolism was differently expressed. These

investigation advanced the understanding of the physiological mechanism of BNS sterility.

The regulatory mechanism of male sterility is still unclear, and the key genes are also still unknown in genic male sterile wheat. We focused on the transcriptional profiling and the role of IAA metabolism in BNS sterile and fertile anthers. An indole metabolic sub-network was found in main metabolic networks involved in differentially expressed genes in almost all paired BNS samples using wheat expression microarray. IAA sub-network contained indole metabolism and alkaloid metabolism nodes. It was not formed in rachis of ear, and could be formed, but incomplete in sterile anthers, in which there was no alkaloid metabolism node. This sub-network was complete in fertile anthers. In F₁ anthers with restorer genes, the sub-metabolic pathway became main subject of different metabolic pathway. More than a dozen key genes were found in the IAA metabolic pathway, four of which were annotated in molecular function. They were Arabidopsis male sterile protein, Arabidopsis 41 KD heat shock protein DanJ, peroxidase and oxalate oxidase precursor. These functional genes and unknown functional genes were inferred to be specifically expressed and constitutively expressed in anther tissues. The complete indole-metabolizing pathway was a relatively independent terminal metabolic pathway with products formation. The intermediate products formed in this pathway could be used to regulate the sterility and conversion of BNS sterility, which may benefit the artificial regulation of BNS sterility and fertility.

Acknowledgements This research was funded by Henan Research Project on Foundation and Frontier Technology (Nos. 122300410011 and 162300410136). We would like to thank prof. ZhenGang Ru, Directorate of Wheat Research Center in Henan institute of science and technology, for supply wheat BNS sterile line. We would also like to thank prof. Gonmbia who give many suggestions for ClueGO running.

Compliance with ethical standards

Conflict of interest The authors have no conflict of interest to declare.

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