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Molecular Evidence of the Haploid Origin in Wheat (*Triticum aestivum* L.) with *Aegilops kotschyi* Cytoplasm and Whole Genome Expression Profiling after Haploidization

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Aegiolops kotschyi cytoplasmic male sterile system often results in part of haploid plants in wheat (*Triticum aestivum* L.). To elucidate the origin of haploid, 235 wheat microsatellite (SSR) primers were randomly selected and screened for polymorphism between haploid ($2n = 3x = 21$ ABD) and its parents, male-sterile line YM21 ($2n = 6x = 42$ AABBDD) and male fertile restorer YM2 ($2n = 6x = 42$ AABBDD). About 200 SSR markers yielded clear bands from denatured PAGE, of which 180 markers have identifiable amplification patterns, and 20 markers (around 8%) resulted in different amplification products between the haploid and the restorer, YM2. There were no SSR markers that were found to be distinguishable between the haploid and the male sterile line YM21. In addition, different distribution of HMW-GS between endosperm and seedlings from the same seeds further confirmed that the haploid genomes were inherited from the maternal parent. After haploidization, 1.7% and 0.91% of total sites were up- and down-regulated exceeding twofold in the shoot and the root of haploid, respectively, and most of the differentially expressed loci were up/down-regulated about twofold. Out of the sensitive loci in haploid, 94 loci in the shoot, 72 loci in the root can be classified into three functional subdivisions: biological process, cellular component and molecular function, respectively.

Keywords: wheat, microsatellite, hexaploid, haploid, gene chip

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Introduction

The production of haploid plants and complete homozygosity double haploids in crop species help accelerate breeding programs, improve selection efficiency and facilitate genetic analysis (Dunwell 2010; Germanà 2011). Although, the significance of haploid in genetics and plant breeding has been recognized for a long time, with the advent of new biotechnology, it has received renewed (Forster et al. 2007) interest. The production of haploids has become an important component of biotechnology programs in many countries (Li et al. 2013).

Haploid can result through natural parthenogenesis or androgenesis. They can also be artificially induced through various techniques including anther culture, microspore culture, unfertilized ovary/ovule culture, distant hybridization and chromosome elimination (Cheng and Korban 2011). Wheat cultivar 'Salmon' produces haploid at a high frequency when its cytoplasm is substituted with that of *Ae. caudate* (Kihara and Tsunewaki 1962). The cytoplasms of five other species of the same genus gave haploid plants at varying frequencies when they were incorporated into the same wheat (Tsunewaki et al. 1974). Sun et al. (1994) embryologically dealt with the process of double fertilization and the traits of embryo and endosperm development in cross of alloplasmic male-sterile wheat K83(21)35A with maintainer line 83(21)35B. They found that the process of double fertilization and the development of embryo and endosperm were similar to those of common wheat and the haploid obviously came from the parthenogenesis of synergid nuclei. Tsunewaki thoroughly studied the mechanism of haploid production in alloplasmic 'Salmon' by means of crosses with morphological marker and specific aneuploid lines, and he hypothesized that besides a cytoplasmic factor two nuclear genes are responsible for the initiation of autonomous embryo formation: a parthenogenesis inducing gene under saprophytic control and a parthenogenesis suppressing gene under gametophyte control (Tsunewaki and Mukai 1990).

Male sterility of wheat with *Ae. kotschyi* cytoplasm (K-type CMS) is considered better than that with *T. timopheevi*. *Ae. kotschyi* contains genes for drought, heat and salt tolerance (Kimber and Feldman 1987). But *Ae. kotschyi* cytoplasm could naturally induce haploid, even in some hybrids between male-sterile wheat crossed with restoring line with the frequency as high as 80% (Kobayashi and Tsunewaki 1980), and the seeds which produce haploid plants looks normal. Cytological observations and epigenetic research all support that the haploid come from maternal parents (Sun et al. 1994). However, both phenotypes and morphological markers were strongly influenced by environmental conditions and development process (Belicuas et al. 2007). Research on the molecular relationship between haploid, male and female parents have, thus, become an interesting field of contemporary research in the production of haploids.

Microsatellite markers are co-dominant and highly reproducible, they are suitable for the analysis of allele segregation in progenies. Biochemical and molecular marker systems are becoming increasingly important today for homozygote testing and they are replacing other techniques, such as self-pollination with subsequent progeny testing and morphological markers (Murovec et al. 2007). Once maternal and paternal microsatellite geno-

types are known, the different pathways for gamete production and unions that give rise to polyploid individuals can be compared for their likelihood. For example, the origin of 2n gametes can be deduced from microsatellite analysis (Nemorin et al. 2013). Analysis of the genetic origin of haploid/diploid regenerates using microsatellite markers has been applied in various fruit crops (Vanwynsberghe et al. 2005; Bouvier et al. 2002), and crops such as the maize (*Zea mays* L., Tang et al. 2006). Muranty et al. (2002) evaluated the homozygosis of spontaneous hexaploid plants derived from anther culture in wheat and triticale (*Tritico secale* Wittmark) by means of microsatellite markers. Their result revealed that most of the spontaneous hexaploid plants were homozygous for all the loci tested and had chromosomes recombining for parental alleles.

Studies on polyploid plants across various genomic scales have occurred relatively more recently, particularly after the whole-genome sequence of *Arabidopsis thaliana* (L.) Heynh became available (Blanc et al. 2003). These studies have vastly increased our knowledge of polyploidy associated genome changes (Adams 2007; Chen 2007). Most results concerning alterations in gene expression after polyploid formation have been obtained from studies in allopolyploids, in which changes to the nuclear environment are more profound than that in autopolyploids (Albertin et al. 2006; Adams, 2007). Autopolyploids also display differences in gene expression relative to their diploid progenitors (Lu et al. 2006). Although gene expression changes are more extensive in studies of polyploidization, we have only limited information about how the extent of change of haploidization (Wang et al. 2011).

The objective of the present study was to use microsatellite analysis to help understand the origin of haploid in K-type CMS line in wheat. Microsatellite markers were genotyped on hexaploid parents and their detected polyploidy offspring. Furthermore, microarray technique was employed to analyse the gene expression at the RNA level between haploids and corresponding hexaploid derived from male sterile line. This work made it possible to identify the origin of haploid, and provided knowledge about whole gene expression profiling after haploidization.

Materials and Methods

Plant materials, RNA extraction, and cDNA synthesis

The haploid wheat ($2n = 3x = 21$ ABD, hereinafter referred to as YM21H) was obtained from a cross between K-type CMS line YM21 ($2n = 6x = 42$ AABBDD, hereinafter referred to as YM21A) and the restoring line YM2 ($2n = 6x = 42$ AABBDD). The maintainer line of YM21A was YM21 ($2n = 6x = 42$ AABBDD, hereinafter referred to as YM 21B). Seedlings were germinated on wet filter paper at 25°C, thereafter vernalized in the dark at 5°C for 18 d, and transplanted to soil (pots of Ø 17 cm) after vernalization. Plants were grown in a greenhouse at 24/18°C (day/night) with a 16 h photoperiod for one or four months.

Shoot and/or root tissues from 1-month-old plants were harvested into liquid nitrogen prior to the extraction of total RNA and genomic DNA (gDNA). Genomic DNA was ex-

tracted from wheat using a modification of the cetyltrimethyl ammonium bromide (CTAB) procedure (Dorrance et al. 1999). Total RNA were extracted using the TRIZOL reagent (Sigma Chemical Co., USA) according to the manufacturer's instructions, and crude RNA preparations were treated with DNase (Invitrogen, USA), following a phenol/chloroform extraction (Sambrook et al. 2001). Complementary DNA was synthesised with Superscript II (Invitrogen, Carlsbad, USA), using oligo (dT) as the polyA primer and following the manufacturer's protocol. To detect the contaminating gDNA, newly synthesised cDNA was amplified with intron-spanning PCR primers (Actin-F: 5'-CTGCTTT GAGATCCACAT-3', Actin-R: 5'-GTCACCACTTTCAACTCC-3'), which could distinguish the contaminating gDNA from the RNA-derived DNA. The entire experiment was performed with three independent biological replicates, and each biological sample was generated by pooling three individual plants together.

Primer selection, PCR amplification, and microsatellite analysis

To investigate the gene expression in haploid and hexaploid wheat, 235 microsatellite primers were randomly selected. RT-PCR was carried out in a volume of 10 µl, which comprised 1 µl cDNA template (1:20 dilution of the cDNA), 5 µl Hotstar Master Mix (Qiagen, USA), 0.25 µl of each primer (10 mM concentration), and 3.5 µl distilled water. The PCR program consisted of a pre-denaturation step of 15 min at 95°C, followed by 35 cycles of 95°C for 30 sec, 59°C for 30 sec, and 72°C for 60 sec, and a final extension step of 72°C for 10 min. Amplicons were electrophoretically separated by microsatellite and visualised by silver staining (Stewart et al. 2011).

Sampling and RNA extraction for Affymetrix gene chip hybridization

One-month-old plants were used to evaluate the differences in gene expression patterns between haploids and its parent, YM21A, with Affymetrix gene chip hybridization. Shoot and/or root tissues of each plant were sampled and frozen immediately, and sent to Genetimes Technology Inc. (Shanghai, China) for cDNA synthesis. Labelled target used in hybridizations was generated from 0.5 µg of total RNA using the One-Cycle Eukaryotic Target Labelling Assay. Hybridizations, washing, staining and scanning were performed as specified in the manufacturer's protocol (http://www.affymetrix.com). The hybridization was performed with three independent biological replicates, and each sample was a pool of three plants.

Affymetrix gene chip data analysis

Fluorescence intensity data were recorded using GenePix Pro v6.0 (Molecular Devices, USA). Lower-quality and weakly hybridizing features were stringently flagged for exclusion (by requiring a circularity ratio ≥ 0.8, signal intensity >75% of background plus one standard deviation, and the sum of median intensity from each channel >900) to initially reduce printing and hybridization errors. Data normalization was performed according to Bolstad's quantile procedure for oligonucleotide arrays (Bolstad et al. 2003). Therefore, control and empty spots were filtered out before normalization. Statistical analysis was

performed with GeneSpring 7.3 (Agilent Technologies) using methods adapted from Casu et al. (2007). Present (P) and Absent (A) calls for Affymetrix probe sets are generated based on statistical analysis of hybridization signal of oligonucleotides from one probe set where Present, $P < 0.04$; Absent $P > 0.06$. GoPipe (Chen et al. 2005) was used for function classification. All the parameters we adopted were recommended by the instruction manual of GoPipe.

RT-PCR validation of microarray results

There are plenty of loci with changed expression pattern during haploidization. According to the different alteration patterns of activation and silence, function, fragment size and expression amount, we chose 10 loci to validate the microarray result with RT-PCR technique (Table S1*). Because the principles were used to statistic the microarray result and real-time fluorescence quantitative RT-PCR data were different, the change fold (the change fold = expression amount in diploid/that in haploid) was chosen to validate the microarray result with RT-PCR technique.

Results

The origin of haploid YM21H

235 wheat SSR primers were randomly selected to provide the polymorphisms between YM21H and its presumed genome donors, of which 200 SSR markers could have amplification products successfully in the haploid and its parents (Table S2). Twenty SSR markers have different amplification products between the YM21H and the restorer line YM 2, and the same differences were noticed between YM21A and YM2 (Fig. S1). On the other hand, among 200 SSR markers we did not find any of SSR markers produced discriminating bands between YM21H and male-sterile wheat YM21A. These 20 SSR markers randomly distributed in wheat chromosome, 10 on A genome, 6 on B and 4 on D (Table S3). Random distribution of 20 SSR markers in wheat chromosome further confirmed that the YM21H does not appear to have genetic materials from YM2. With the results that no differences of amplified products of 200 markers between the YM21H and the maternal plants, we show the molecular evidence that the haploid was originated from maternal parent.

The sperm cells fertilized the polar nuclei forming a primary triploid endosperm cell which subsequently develops into endosperm, normally, in the same time another sperm cell fertilized the egg cell, forming the diploid zygote. Using pressed disc method a haploid was identified from sampling of four-day seedlings. As shown in Fig. S2, we cautiously separated endosperm from seedling and extracted DNA from them, respectively. PCR analysis with specific probe for HMW-GS showed that 1Ax1 subunit presented in paternal parent YM2, but not exist in maternal parents YM21A. As expected, the endosperm and seedlings from hexaploid offspring in the cross between YM21A and YM2 all

* Further details about the Electronic Supplementary Material (ESM) can be found at the end of the article.

could detect 1Ax1 subunits. But the 1Ax1 subunits were only found in YM21H endosperm, while we could not detect them in YM21H seedlings. The results demonstrated that the endosperm was fertilized products (polar nuclei was fertilized) but the embryo produced without fertilization, that indicated further the haploid was produced without fertilization and explained why the haploid seed looks normal and without obvious difference with the seeds of hexaploid.

Gene expression in haploid and the ploidy sensitive loci

To understand the whole gene expression in haploid, we performed a global analysis of gene expression using the Affymetrix Gene Chip and the microarray results were validated by RT-PCR. As is shown in Table S4, 85% of the loci had the similar expression patterns derived from microarray data and RT-PCR result.

Only those loci with expression alteration of twofold or more were chosen for analysis. The locus with an expression increase in haploid for over twofold is defined as expression up-regulation, or reversely, as down-regulation. After haploidization, a total of 1051 loci (1.7% of all the probes on the chip) in shoot, were detected to alter their expression patterns, including activation, up-regulation, silence and down-regulation. On the contrary, only 0.91% of total sites changed for expression in root. We call those loci with an altered expression pattern as Ploidy Sensitive Loci (PSL). In order to clearly detect the gene expression patterns between the tissue of shoot and root, the activated and silenced loci (signal as A-P, P-A) were excluded in present analysis. Some loci that expression was not obvious (signal as M-P, P-M) were also removed from PSL. As is shown in Fig. S5, the PSL had different expression changed fold, 2–12 for down-regulation, 2–7 for up-regulation and 2–3 for both up- and down-regulated in the shoot tissue of YM21H. The corresponding changes were 2–7-fold for down-regulation, 2–8-fold for up-regulation and 2–3-fold for both up- and down-regulated in the root tissue of YM21H. Loci with expression alteration of twofold accounted for 69.87% of total down-regulated loci, 82.37% for up-regulated in the shoot tissue of YM21H. Loci with expression alteration of twofold were accounted for 71.01% of down-regulated loci, 84.73% for up-regulated in the root tissue of YM21H, respectively.

The function classification of ploidy sensitive loci

Out of the 760 PSL in shoot of YM21H, 94 (12.37%) can be classified into three functional subdivisions: biological process, cellular component and molecular function (Tables S5–S7). On the contrary, out of the 403 PSL in root of YM21H, 72 (17.87%) can be classified into these three functional subdivisions. Because some sequences had several functions, they can be classified into more than one type, resulting in that the loci sum in all functions were more than 94 in shoots, 72 in roots, respectively.

Out of the 94 loci with the predicted function, 63 involve the biological process in shoots of YM21H. Among them, most (42.86%) belong to metabolism. Out of the 72 loci with the predicted function, 53 involve the biological process in root ofYM21H, and also most (47.17%) of them belong to metabolism (Table S5).

Out of the 94 loci with the predicted function, 69 involve the molecular function in shoots of YM21H. Among them, the ratio of the loci with catalytic activity is the highest (43.48%). The secondly highest ratio (36.23%) is those with the binding function. In contrary, out of the 72 loci with the predicted function, 56 involve the molecular function in roots of haploid. Among them, the ratio of the loci with catalytic activity is the highest (53.57%). The secondly highest ratio (53.57%) is those with the binding function (Table S6).

Out of the 94 loci with the predicted function, 52 involve the cell component in shoots of YM 21H. Among them, the ratio of the loci with cell structure is the highest (26.92%). The secondly highest ratio (15.09%) is those of intracellular components. On the contrary, out of the 72 loci with the predicted function, 20 involve the cell component in roots of haploid. Among them, the ratio of the loci with cell structure is the highest (90.01%). The secondly highest ratio (25.09%) is those of intracellular components (Table S7).

Discussion

As for the origin of haploid, most studies focused on cytological observations and epigenetics research, however, there is no molecular evidence to prove their findings. The data set from our study is unique and valuable considering the following points. Same amplification patterns of 235 SSR markers randomly selected strongly confirmed that YM21H has the same genetic background with maternal parent. The microsatellite profiles obtained on the restorer lines YM2 and YM21H indicate that haploid has no known genetic materials of paternal genome. The distribution patterns of HMW-GS among haploid and their parents further confirm our hypothesis. The present study clearly established that the production of haploid was a moderate process. Naturally occurring haploid was an excellent material, which can increased our knowledge of polyploidy associated genome changes. Further microarray analysis revealed that the differentially expressed loci were up- and down-regulated mainly about two–threefold. What is the most important, the regulation mechanisms driving those changes in gene expression in shoot was obviously differently with that in root after haploidization.

YM21H originate spontaneously from maternal parentsYM21A

We randomly selected 235 pairs SSR primers to evaluate the origin of haploid. Among them, 76% of SSR markers yielded identifiable polymorphism, however, we did not find any different bands between YM21H and maternal genome. Moreover these SSR primers were in tandem and randomly spread in wheat genomes. Such evidence strongly revealed that YM21H and YM21A have similar genetic background. On the other hand, 20 (8%) pairs of SSR markers have significantly different amplification products between YM21H and YM2. The different amplification products between YM21H and restore lines YM2 made it possible to confirm that there were no genetic materials in haploid coming from paternal genome.

1Ax1 subunit of HMW-GS presented in restore line YM2, but not inYM21A. Further analysis showed that either endosperm or seedlings of hexaploid offspring were all have

1Ax1 subunit. On the contrary, 1Ax1 subunit can be found only in haploid endosperm but not detect in their seedlings. Account for that endosperm and seedlings were separated from the same haploid seeds, which produced from the cross between YM21Aand YM2, We could concluded that a single fertilization happened in the process of fertilization, endosperm was fertilized production and embryo developed without fertilization.

In the case of haploidization, this molecular marker analysis provides sufficient evidence of the haploid origin of maternal parents, because there is no other possible explanation for the occurrence of haploid structures from donor plants. Based on the data from this study and previous cytological observations and epigenetic research, we supposed that the haploid YM21H was naturally produced by male sterile wheat YM21A.

The differentially expressed loci were up- and down-regulated mainly about twofold

Although gene expression changes are more extensive in studies of polyploidization, limited amount of research has been done to investigate the genome changes in haploidization. In this experiment, microarray analysis revealed there was 1.7% in shoots, 0.91% in roots of all the probes on the chip was detected to alter their expression patterns after haploidization, respectively. This contrast with the high proportion of 5% of genes in newly synthesized wheat hexaploids (Kashkush et al. 2002) was silenced. The proportion of PSL were also lower than that in SARII–628 rice *(Oryza sativa)* haploid (2.47%) (Zhang et al. 2008), and this may due to the diploid property of rice. Further analysis revealed that among these twofold differentially expressed loci, 69.87% of that was down-regulated in the shoot tissue of haploid, and 82.37% up-regulation in the shoot tissue, 71.01% down-regulation, and 84.73% up-regulation in the roots tissue of haploid, respectively. In conclusion, the differentially expressed loci were up-regulated and down-regulated mainly about twofold after haploidization.

The strategies of genetic regulation in shoot tissue and root tissue were different

The growing amount of gene expression data in polyploidy research has stimulated scientists to speculate the regulation mechanisms driving those changes in gene expression (Chen and Ni 2006). However, to date, the experiment data to support their speculation are still very limited (Zhang et al. 2008). Haploid has only one set of genomes and must adjust more on gene expression for the sake of survival. Based on the relationship between gene expression and the chromosome or chromosome arm duplication, some scientists proposed a dosage effect or dosage compensation effect to understand ploidy effects on gene expression (Guo et al. 1996). As shown in Fig. S3, the number of up-regulated loci was significantly more than that of the down-regulated loci in shoots. It is reasonable for haploid to preferably increased genes expression because this can reinforce the expression of favourable traits and functions and weaken the influence of unfavourable factors on survival. However, as we did not expect, the number of up-regulated loci was less than that of the down-regulated loci in shoots of haploid. This deviation in root tissue and shoot tissue suggest that the regulation mechanisms of gene expression were variable in different haploid organ.

Among the loci with the predicted function involve the biological process in haploidization, either in shoot or in root the ratio of the loci for metabolism is the most. The loci for DNA and RNA metabolism are very important because the former is necessary for genome number shift and the latter is directly related to the following analysis on expression alteration. Because the alteration on cell size is also the typical characteristic of ploidy shift, the gene related to cell growth/maintenance shows a certain variation in haploidization. Moreover, the cell size variation was also considered to cause the expression alteration in a yeast homologous ploidy series (Galitski et al. 1999). However, as shown in Table S5, 26.92% loci involve the cell structure in shoot of haploid. On the other hand, 90.01% loci involve the cell structure in root of haploid. The regulation mechanisms driving those changes in gene expression in shoot was obviously differently with that in root tissue in haploidization.

Wheat is one of the most important food crops in the world, and transcriptomics studies of this crop promise to reveal the expression dynamics of genes that control many agriculturally important traits. When working with plants that are normally polyploid, it is very useful to work at a low ploidy level through haploid induction (Carputo and Barone 2005). The establishment of completely homozygous lines of a selected species in one generation without the need of self-pollination for several years enables numerous applications of haploids and DHs, such as the production of homozygous lines, induced mutations, chromosome reduction of polyploid species, gene or quantitative trait loci (QTL) mapping, genomics, and transformation experiments (Murovec et al. 2007). As a result of haploid induction followed by chromosome doubling, homozygosity can be achieved in the quickest possible way making genetic and breeding research much easier. This study revealed a certain ratio of down-regulated or up-regulated loci after YM21A haploidization. What traits are controlled by those down-regulated or up-regulated? How does haploid regulate the down-regulated or up-regulated for survival? Further studies are needed to probe those questions.

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Electronic Supplementary Material (ESM)

Electronic Supplementary Material (ESM) associated with this article can be found at the website of CRC at http://www.akademiai.com/content/120427/

Electronic Supplementary *Table S1*. Primers used in real time quantitative RT-PCR analysis

Electronic Supplementary *Table S2*. Screening of 235 pairs SSR primer

Electronic Supplementary *Table S3*. Chromosome locations of 20 polymorphic markers

Electronic Supplementary *Table S4*. RT-PCR amplification with different primers in haploid and hexaploid

Electronic Supplementary *Table S5*. GoPipe function categories according to biological process

Electronic Supplementary *Table S6.* GoPipe function categories according to molecular function

Electronic Supplementary *Table S7*. GoPipe function categories according to cell components

Figure S1. Amplification patterns of the haploid and its parents with 20 polymorphic SSR markers

Figure S2. Analysis distribution of HMW-GS in haploid and its parents

Figure S3. Expression changed folds of haploidization-sensitivesites in shoots and roots