Efficient development of practically usable thermo-photo sensitive genic male sterile lines in wheat through doubled haploid breeding

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Abstract

Background: Two-line hybrid wheat system using thermo-photo sensitive genic male sterility (TPSGMS) is now a dominant and promising approach of wheat heterosis utilization in China. However, during past twenty years only several TPSGMS lines have been capable of practical application in hybrid wheat breeding and production, which reduced the opportunities and efficiency of creating hybrids with strong heterosis. Introducing doubled haploid (DH) breeding could be a helpful strategy to efficiently develop practically usable TPSGMS lines.

Results: F 1 s and selected F 2 and F 3 sterile plants from eight crosses made from two commercial TPSGMS lines were used to produce DH lines by using the wheat × maize system. Twenty four elite sterile lines possessing stable sterility, good outcrossing and yield potential, resistance to yellow rust and powdery mildew, and desirable plant height (50-60 cm) were obtained within 4 years through at least one year evaluation. Twenty from twenty four elite lines showed stable sterility in repeated tests of two or three years, will be selected for hybrid breeding. The percentage of elite lines within total tested DH lines produced from filial generations was in the order of F 2 > F 3 > F 1 in this study.

Conclusions: Our study shows that DH breeding is more efficient for the selection of traits controlled by recessive gene(s) compared with conventional breeding, especially for the sterility of TPSGMS wheat. Coupling DH techniques with conventional breeding would be an efficient strategy for developing practically usable wheat TPSGMS lines in respect to number and saving time, which is helpful for further improving the efficiency of wheat hybrid breeding. Producing DHs from F 2 generation appeared to be the better choice considering the balance of shortening breeding time and overall breeding efficiency.

Background
Wheat provides about 20% of the world’s daily supply [1]. Heterosis utilization in wheat is one of the effective ways for further increasing yield potential and stability, which is of great importance for increasing the productivity of wheat to meet the growing demand [2-4]. However, how to develop hybrids with super heterosis and produce hybrid seeds efficiently remains a worldwide problem during past half a century[1, 4].

Unlike hybrid wheat systems based on cytoplasmic male sterility (CMS)[5] and photoperiod-sensitive cytoplasmic male sterility (PCMS)[6], the two-line hybrid wheat system using thermo-photo sensitive genic male sterility (TPSGMS) is a new way of wheat heterosis utilization in China. The TPSGMS line is characterized as being sterile under low-temperature and short-day for hybrid seed production, and fertile under high-temperature and long-day for self propagation. Therefore, this system does not need the maintainer line, can use wide resources of restorer lines and makes hybrid seed production procedures easier [7-9]. From 2002 to 2018, 20 hybrid wheat varieties released in China, 14 were from TPSGMS two-line system and applied in China with yield increase of 10-15%, especially in poor lands [10-12]. Meanwhile, encouraging multi-location demonstrations have been achieved in Vietnam, where “Yunza” hybrid varieties performed much better than local inbred cultivars in yield, drought tolerance and fertilizer input [13]. However, 14 TPSGMS-based hybrid varieties only had about 0.5% of total 2,691 wheat varieties released in China from 2002 to 2018 [14], and few hybrid varieties were applied in main producing areas such as Yellow-Huai River wheat zone of China, where inbred varieties perform well in yield while most hybrid varieties available did not exhibit enough yield advantage. An important cause for such situation is that less than ten practically usable TPSGMS lines across China were available over past 20 years, which greatly reduced the opportunities and efficiency of creating superior hybrids even ten thousands of restorers were test-crossed. In contrast, 106 rice TPSGMS lines and their 1,074 hybrid varieties
were separately released during the same period in China [14]. Therefore, more wheat TPSGMS lines usable for developing hybrid variety with strong heterosis as well as efficiently producing qualified hybrid seeds should be developed and utilized.

Doubled haploid (DH) techniques that can make any segregating material homozygous in one generation, have been widely used in crop breeding to improve the efficiency of selection and accelerate the breeding process [15-19]. The DH technique using the intergeneric cross between wheat (*Triticum aestivum* L.) and maize (*Zea mays* L.) has become an integral part of many commercial wheat breeding programs as DH lines offer economic, logistic and genetic benefits over conventional inbred lines, with advantages of stable induction rate and few genotypic restrictions [20-21, could be helpful for improving development of wheat TPSGMS lines.

This study aimed to evaluate the efficiency of developing wheat TPSGMS lines by using DH technique based on wheat × maize in breeding program with sterile materials derived from different filial generations of F$_1$, F$_2$ and F$_3$.

**Results**

**Production of DH lines**

During summer sowings of 2014-2016, we produced DHs with four F$_1$s and sterile plants selected from F$_1$ and F$_2$ segregating populations by wheat × maize system (Table 2). A total of 920 DH lines were obtained from all eight crosses (Table 3). In Dec. 2016, a serious frost damaged some doubling treated plants that were heading, resulting in partial failure to obtain DH seeds. Variance analysis showed that there were significant difference in embryo rate (P=0.00) and haploid seedling rate (P=0.00) among different combinations, which indicated that embryo rate and haploid seedling rate were more susceptible to genotypic influence. The averages of embryo rate, seedling rate of embryos and
chromosome doubling rate of seedlings were 36.76%, 62.65% and 86.42% respectively, exhibiting good efficiency in DH production as showed in our previous study [22]. Temperate climate at Kunming especially from May to October allows planting of spring and vernalized winter wheat materials throughout the year under natural conditions here (Fig. 1 and Supplemental Figure 1), which facilitates mass production of wheat DHs by wheat ’ maize crosses because plenty of fresh pollens are available from naturally and repeatedly planted maize plants from late April to early November [22-26].

**Selection of candidate DH sterile lines**

All 920 DH lines produced from F₁, F₂ and F₃ generations were separately tested for sterility during 2016-2018. Among them, 295 DH lines showed normal seed set were excluded from further testing. These lines were mainly from F₁ generations as expected.

In the 1ˢᵗ (Oct. 15) and 2ⁿᵈ (Nov. 20) sowings, 210 lines (33.60%) and 66 lines (10.65%) of 625 DH lines in total tested had seed setting rates less than 5%, respectively. 41 (6.56%) DH lines showed seed setting rate less than 5% in both sowings (Table 4, Fig. 2 and Supplemental Figure 2). When keeping in view other desired traits of out-crossing rate (≥70%), disease resistance (to stripe rust, powdery mildew), plant height (50-60 cm), tillering and spike formation ability, 24 elite lines were finally selected from 41 lines above mentioned.

When sterile lines were sown on Oct. 15 (1ˢᵗ sowing) and Nov. 20 (2ⁿᵈ sowing), their sensitive periods (causing fertility alteration) would be the dates from middle to late February and from late March to early April, respectively. Consequently, during sensitive periods, the 1ˢᵗ sown sterile lines would have low temperature and short-day to fully exhibit sterility, while the 2ⁿᵈ sown lines would have relatively higher temperature and longer day length that cause the early heading spikes sterile and the late heading tillers
partially fertile to produce a few seeds for propagation (Fig. 1 and Supplemental Figure 1).

According to our experiences, TPSGMS lines that exhibit 100% sterility in the 2\textsuperscript{nd} sowing date are usually stable in sterility but difficult in propagation, which make them not suitable for practical application. In southwest of China wheat is normally sown from middle Oct. to early Nov., thus a TPSGMS line with seed setting rate <5% in both sowing dates (from Oct. 15 to Nov. 20) would be able to meet the demand for safe production of qualified hybrid seeds.

**Sterility determination of DH lines derived from F\textsubscript{1} generation**

Ten elite DH sterile lines derived from F\textsubscript{1} were repeatedly tested during 2016-2018. In 2016/2017, the seed setting rates of all lines were 0 in the 1\textsuperscript{st} sowing date, and ranged from 2.98\% to 4.87\% in the 2\textsuperscript{nd} sowing date (Table 5). In further test of ten sowing dates in 2017/2018, the seed setting rates of the ten elite lines were < 1\% from the 1\textsuperscript{st} to the 3\textsuperscript{rd} sowings (Oct.22-Nov. 5), < 5\% till the 5\textsuperscript{th} sowing (Nov. 19), and ≥ 50\% in the 10\textsuperscript{th} sowing (Dec. 24), suggesting sowings before Nov. 5 and Nov. 19 were separately the optimum and suitable times for hybrid seed production while after Dec. 24 the suitable time for propagation of these sterile lines (Fig. 3 and Supplemental Figure 3).

During 2016-2018, ten elite TPSGMS lines derived from F\textsubscript{1} generation showed highly or 100\% sterility in three years when sown from Oct. 15 to Nov. 5, although the average temperatures varied from 12°C to 15°C during their sensitive periods from the second half Feb. to the first half Mar. (Fig.1 and Supplemental Figure1), will be utilized in hybrid breeding later.

**Sterility evaluation of sterile lines derived from F\textsubscript{2} generation**

Ten selected lines in 2016/2017 derived from F\textsubscript{2} were tested again in winter sowing of
2017/2018. The seed setting rates of all lines were 0 in the 1st sowing, and ranged from 1.99% to 4.04% in the 2nd sowing (Table 6). All elite lines showed stably in sterility during two-year cycles, and will be planted in ten sowing dates for further determination in sterility, suitable times for hybrid seed production and propagation. Meanwhile, preliminary test-crosses will be conducted with these lines.

**Evaluation of out-crossing potential for elite TPSGMS lines**

In winter sowing of 2018/2019, the out-crossing potential of 20 elite TPSGMS lines derived from F\textsubscript{1} and F\textsubscript{2} generations were evaluated. The out-crossing rates of 20 lines ranged from 70.46 % to 93.90% with average of 82.87%. There were 13 lines including 8 derived from F\textsubscript{2} generation with out-crossing rate > 80%, 4 lines between 75% and 80% and 3 lines between 70% and 75% (Table 7). All 20 lines showed high out-crossing potential even only one round of selection was done after DH production, because doubled haploids had ‘genetically fixed’ the trait, which confirmed our previous results[27]. However, more lines derived from F\textsubscript{2} generation appeared to have better out-crossing ability (>80%) compared with that from F\textsubscript{1} generation, suggesting one more cycle of selection before DH production would help to further concentrate the target trait. In fact, the results of out-crossing rates here were obtained by pollination with nearly unlimited pollen supply, and need to be further assessed in practical hybrid seed production.

**Breeding efficiency of different generations**

According to seed setting rates < 5% in both sowing dates, 41 DH sterile lines including 13, 15, and 13 lines separately derived from F\textsubscript{1}, F\textsubscript{2} and F\textsubscript{3} generations were selected, with breeding efficiency (percentage of selected DH lines in total DH lines tested) of 4.14%, 7.35% and 12.15% in F\textsubscript{1}, F\textsubscript{2} and F\textsubscript{3}, respectively. When out-crossing ability, resistance to diseases and other desired traits were further considered, 24 elite lines were left and the
overall breeding efficiency in F₁, F₂ and F₃ were 3.18%, 4.90% and 3.74%, respectively. U-test analyses indicated that there were significant differences (P<0.01) in breeding efficiency of producing DHs from F₁, F₂ and F₃ generations (Table 4). The trend of breeding efficiency for a single trait (sterility) was in the order of F₃ > F₂ > F₁, while for comprehensive traits was F₂ > F₃ > F₁.

Discussion

Utilization of male sterility is the basis of commercial application of hybrid wheat. It’s common for all genetically controlled sterility systems that the more sterile lines capable of commercial utilization are used for test-crossing, the more opportunities of creating hybrids with super heterosis would present. Though the TPSGMS based two-line hybrid system was established as early as 1990S [7, 28] but less than ten TPSGMS lines capable for commercial usage have been developed and utilized up to now in north and south wheat zones of China. Pedigree method is commonly used in developing TPSGMS lines [29, 30], however, there were some difficulties hindering the breeding efficiency. The sterility of TPSGMS line is controlled by recessive nuclear major genes plus minor genes[7, 31-34], causing a very low proportion of highly sterile plants in segregating populations, especially in F₂s derived from crosses between sterile lines and normal fertile lines. When other important traits like outcrossing ability, plant height, yield potential, disease resistance, etc. are considered together, the breeding efficiency would become extremely low. Theoretically, the probability of homozygous recessive individuals in F₂ population is 1/4ⁿ, while that in DH population produced from F₁ would be 1/2ⁿ, suggesting DH breeding is more efficient for selection of traits controlled by recessive genes, especially for the sterility of TPSGMS wheat. Also, crossing between semi-sterile materials and sterile lines further increased the proportion of highly sterile plants in segregating populations of this
study, which is similar in effectiveness to backcrossing with sterile lines [29].

Meanwhile, few effective molecular markers are currently usable for marker assisted selection in sterility [35]. Consequently, it would take long time to develop a genetically stable TPSGMS line because the expression of sterility in TPSGMS lines needs restricted temperature and light conditions which could be found only once a year[30]. In our previous breeding program, only two TPSGMS lines K78S and K456S capable of commercial usage were developed by pedigree methods from 1996 to 2010, while in this study we developed 24 elite TPSGMS lines with complete homozygosity and other desired traits within 4 years by introducing DH techniques.

Another issue we address is to identify the better generation for producing DHs. Most breeders prefer to produce DHs from F\textsubscript{1} generation to shorten the breeding cycle, but it may limit the opportunity for recombination [15]. Therefore, producing DHs with selected individuals from F\textsubscript{2} generation of single crosses or F\textsubscript{1} generation of pyramiding crosses seems better than that from F\textsubscript{1} generation of single crosses [36]. Similarly, Snape and Simpson (1981) inclined to produce DHs from F\textsubscript{2} generation in barley by comparing the gain in genetic variation for 6 agronomic traits with DH lines derived from F\textsubscript{1}, F\textsubscript{2}, F\textsubscript{3} and intermated F\textsubscript{2} (S3) generations [37]. In contrast, Iyamabo and Hayes (1995) did not found more favorable genotypes in DH lines produced from F\textsubscript{2} generation than that from F\textsubscript{1} generation in barley, therefore, they preferred to use F\textsubscript{1} generation for producing DHs [38]. In present study, the overall breeding efficiency of producing DHs from filial generations was in the order of F\textsubscript{2} > F\textsubscript{3} > F\textsubscript{1}, indication F\textsubscript{2} generation is better for producing DHs in breeding efficiency, which confirmed the majority results above. However it still needs to be further investigated by comparing the breeding efficiency of producing DHs with F\textsubscript{1} and selected plants of F\textsubscript{2} and F\textsubscript{3} derived from the same cross.
Producing DHs from F₁ generation had less breeding efficiency because only one round of recombination occurred and no selection was done. As a result, a high frequency of agronomically undesirable lines were produced [37], which was confirmed in this study as most fertile lines discarded came from F₁ generation. However, using F₁ generation for DH breeding has the edge in saving time, it could be useful for crosses with better predictability and coping with urgent needs for developing varieties with resistance to diseases such as yellow rust for its frequently varying pathogenic races.

Conclusion

In this study we developed at least 20 practical TPSGMS lines of wheat that showed stable sterility in two or three years tests, high outcrossing potential and other desirable traits within 4 years, which verified that DH breeding is more efficient for the selection of traits controlled by recessive gene(s) compared with conventional breeding, especially for the sterility of TPSGMS wheat. Introducing DH technique is an efficient strategy in developing TPSGMS lines of wheat, both in number and saving time. Generally, producing DHs from F₂ generation appears to be the better choice with balance of breeding efficiency and shortening of breeding cycle. However, this result should be further investigated by using diverse genetic materials of different filial generations derived from the same combinations. More practically usable TPSGMS lines would further improve the efficiency of wheat hybrid breeding by increasing the opportunity of developing hybrids with high heterosis.

Methods

Plant materials

Two TPSGMS lines and five semi-sterile advanced lines of wheat were used in the study (Table 1). A maize variety “Baitiannuo” was used as pollen donor in DH production. All
wheat and maize materials were bred by Institute of Food Crops, Yunnan Academy of Agricultural Sciences, Kunming, China.

**Crossing and DH production**

Wheat materials were late sown in January 2014 to make semi-sterile materials fertile for crossing with sterile lines K78S and K456S at Kunming, Yunnan province, China (25°02'N, 102°42'E, altitude 1960 m), where spring wheat could be planted and harvested throughout the year. For DH production, maize sowing (in April) began two months before wheat sowing (in June) to synchronize their flowering dates, and maize was sown in three dates with interval of 14 days. The crossings and handling of subsequent generations are summarized in Table 2.

Before producing doubled haploids, pedigree selection was adopted to select sterile plants from segregating population of F$_1$ and F$_2$ generations according to performances in sterility, out-crossing potential including glume opening and stigma exertion [27], plant height (50-60 cm), resistance to yellow rust and powdery mildew, tillering ability and the yield potential. Seeds of sterile plants were harvested from regenerated tillers by cutting all spikes of sterile plants followed by intensive irrigation and fertilization.

**Method of producing DHs**

For DH production, an improved protocol [22, 23] was adopted. Wheat spikes were pollinated with fresh maize pollen 24 to 48 hours after emasculation. Pollinated tillers were cut 24 hours after pollination and sprayed with 100 ppm 2,4-D, then cultured in growth chambers for 14 days with nutrition solution containing 100 mg L$^{-1}$ 2,4-D, 40 g L$^{-1}$ sucrose, 10 mg L$^{-1}$ silver nitrate, 3 g L$^{-1}$ potassium dihydrogen phosphate and 3 g L$^{-1}$ urea, keeping a regime of 10 h light/14 h darkness with light intensity of 6000 Lux, constant temperature of 25°C and relative humidity of 80% in the chamber. Nutrient solution was
replaced every three days. Embryos were aseptically dissected from 14-day caryopses and cultured on half-strength MS medium [39] under darkness at 24-25°C until germination, then moved to the growth house at a regime of 10 h light/14 h darkness, with light intensity of 3000 Lux and a constant temperature of 25°C and humidity of 75%. When seedlings developed two to three tillers, the plants were taken out and immersed in 0.05% colchicine solution for 8 h at 25°C to induce doubling of chromosomes. Treated seedlings were transplanted into pots to grow until booting stage, then moved into greenhouse for 15 days, keeping temperature > 20°C to ensure fertility of DH plants. All plants were bagged before flowering and harvested one by one.

**Sterility evaluation of DH lines**

DH lines obtained during 2015-2017 were first evaluated by sowing at two dates on Oct.15 and Nov. 20, respectively. Lines with high sterility were kept for further evaluation. At least 10 spikes per line in each sowing were randomly bagged before flowering to measure the seed setting rate and out-crossing potential [27]. Other important traits such as disease resistances and yield potential were also recorded. Selected lines in 2015/2016 and 2016/2017 were again evaluated in 2016/2017 and 2017/2018. We also conducted a ten-sowing assessment from Oct. 22, 2017 to Dec. 24, 2017 with an interval of 7 days for elite DH lines from F₁ generation that were selected in last two-year tests. The TPSGMS line K78S was used as the check in all tests. The seed setting rate (SSR) was calculated following Yang et al. (2006)[31]:

\[
SSR (\%) = \frac{gn}{sn \times 2} \times 100
\]

Where \( gn \) means the number of grains from bagged spikes, \( sn \) the number of spikelets. A TPSGMS line with SSR < 5% was recognized as highly sterile and qualified for hybrid seed production [31].

**Out-crossing test of DH lines**
Twenty elite DH sterile lines derived from $F_1$ and $F_2$ generations were separately planted as 10 rows in a plot of 1 m × 2.5 m surrounded by about 600 restorers on Oct. 15, 2018. Open pollination was aided by natural wind of grade 3-6 which is usual in Yunnan throughout wheat growing seasons. Twenty spikes for each line were randomly bagged before flowering. Twenty open pollinated spikes were randomly harvested from 10 rows of each line with the bagged spikes to measure the out-crossing rate (OR) of sterile lines following [27, 31]:

$$ OR (%) = \frac{(ugn-bgn)}{(sn \times 2)} \times 100 $$

Where $ugn$ and $bgn$ are un-bagged and bagged grain numbers at two basal florets of each spikelet respectively, $sn$ the number of spikelets per spike. $bgn$ is counted to exclude the possible self-pollination seed setting because TPSGMS lines are not always keeping 100% sterile.

**Yellow rust and powdery mildew scoring**

Yellow rust and powdery mildew occur every year at Kunming, and highly susceptible cultivar of these two diseases were planted close to tested sterile lines as control and spreaders. So the resistance of sterile lines to the diseases was scored in the field according to Han et al (2010) for yellow rust [40] and Li et al for powdery mildew [41].

**Data collection of temperature and day-length**

Data of temperature during 2016-2018 were collected from a data-logger ‘HUATO S100-TH’ in thermometer screen near the field, and daylengths from the meteorological station of Kunming.

**Statistical analysis**

The data of embryo rate, haploid seedling rate and chromosome doubling rate were analyzed using one-way analysis of variance (ANOVA) followed by Fisher's least significant difference (LSD) test. U-test of multiple percentage comparison [42] was conducted for
evaluating the significant differences in breeding efficiency of producing DHs from F$_1$, F$_2$ and F$_3$ generations. SPSS and Excel Office were used in statistical analyses.

Abbreviations

TPSGMS: thermo-photo sensitive genic male sterility; DH: doubled haploid; SSR: seed setting rate; gn: grain number of bagged spikes; sn: spikelet number; OR: out-crossing rate; ugn: un-bagged grain numbers at two basal florets of each spikelet; bgn: bagged grain numbers at two basal florets of each spikelet; NC: number of caryopsis; NE: number of embryos; NHS: number of haploid seedlings; NDH: number of double haploids; RIE: rate of immature embryos; SR: haploid seedling rate; CDR: chromosome doubling rate.

Declarations

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Authors’ contributions

HS Li and SX Li conceived and designed the research; S Abdelkhalik, A Shahzad, J Gu and H Zhao performed the experiments; HS Li, A Shahzad and MJ Yang wrote the manuscript; ML Ding and K Liu contributed to preparation of the manuscript; All the authors read and approved the manuscript.

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analysis, and interpretation of data and in writing the manuscript.

**Availability of data and materials**

All data and materials generated or analyzed during this study are included in this article or are available from the corresponding author on reasonable request.

**Ethics approval and consent to participate**

Not applicable.

**Consent for publication**

Not applicable.

**Competing interests**

The authors declare that they have no competing interests.

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Tables

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Figures

Figure 1

Daily average temperature and day length around the year during 2016-2018.

Jan.1 means the first half of the January, Jan.2 means the second half of the January, the same as that of other months. ■, □, ×: mean average temperatures of every half month from January 1, 2016 to September 30, 2018; ○, ◇: mean average daylengths of every half month during the same periods.
Figure 2

Seed setting rate distribution in first and second sowings of 314 DH lines in 2016 (A), 204 DH lines in 2017 (B), and 107 DH lines in 2018. First and second sowing dates: October 15 and November 20 of every year, respectively.

Figure 3

Seed setting rates of ten elite TPSGMS lines in ten sowings of 2017/2018 cycle. Sowing dates started from October 22, 2017 with interval of seven days.

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