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Extra-Large Seed Germplasms of *Brassica Napus* Created through Microspore Culture

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Abstract: Thousand Seed Weight (TSW) is one of the major yield components of rapeseed (*Brassica napus* L.). Here reports an extra-large seed germplasm GM01 which was obtained through isolated microspore culture. Three-way cross was made: H8—a Yunnan spring early-maturing rapeseed variety, "Legency"—a Canadian canola variety and "020010"—a semi-winter late-maturing rapeseed variety. One hundred and forty eight doubled hyploid lines were obtained from the F_1 plants of three-way cross through isolated microspore culture. Among them, the TSW of GM01 amounted to 8.68 g and the TSW of 53 lines were above 5.0 g. The TSWs of GM01 were relatively stable among the multi-location field trials from 2007 to 2014 with variation being only 10%-15% among the locations and years. Compared with H8, GM01 had larger flowers, stigmas, siliques and seed diameters, but less branches, siliques per plant and seeds per silique.

Key words: *Brassica napus*, germplasm, seed weight, morphological trait, microspore culture.

1. Introduction

Rapeseed (*Brassica napus* L.) is one of the important oil crops for edible plant oil, other staples, such as rice, maize and wheat, which continuously improve the seed yield is always the major objective for rapeseed breeders. Rapeseed yield per unit area is determined by two main components, that is, the number of seeds per unit area and the seed weight [1, 2]. The number of seeds per unit area is closely related to the number of branches per plant, the number of pods per plant and the number of grains per pod [3-5]. Therefore, high yielding can be obtained by increasing either the number of seeds per plant, 1,000-seed weight (TSW) or the both [6, 7]. At present, the yield of main cultivated varieties of rapeseed was about 2,500-4,000 kg/hr and TSWs of the varieties were in the range of 3.0 g to 3.5 g [1]. According to the assumption of Berry, P. M. [1], in order to obtain the seed yield of 6,000 kg/hr, the TSW of cultivar should reach 5.0 g and for the seed yield of 9,000 kg/hr, TSW of should be up to 6.10 g. Thus, high TSW should be one of the most important characteristics of excellent variety.

Germplasm resources are the material foundation for crop breeding. Rapeseed (AACC, 2n = 38) is allopolyploid. It originates about 0.12-1.37 million years ago in the Mediterranean region by natural hybridization between *Brassica rapa* (AA, 2n = 20) and *Brassica oleracea* (CC, 2n = 18) [8, 9], and has a short domestication history of only 400-500 years [10, 11]. Because of its high yield and wide adaptability, rapeseed replaces *Brassica rapa* and *Brassica juncea* and becomes the plants worldwide gradually. Comparing to *Brassica rapa* and *Brassica juncea*, rapeseed has short evolution history and intensive selection in the traditional breeding process, which resulted in its poor genetic diversity. The poor genetic

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diversity has become the bottleneck of rapeseed breeding and utilization of heterosis [12, 13]. According to the statistics of International Board for Plant Genetic Resources (IBPGR), there were about 43,000 accessions of *Brassica* germplasm. In these accessions, *Brassica rapa*, *Brassica juncea* and rapeseed accounted for 34.09%, 27.27% and 14.32% of total accessions respectively. In 1996 to 1999, Liu, H. L. [14] collected 871 rapeseed accessions and *Brassica juncea* germplasms from the western region of China. Among these accessions: the TSWs of the wild germplasm were less than 1.0 g; the average TSWs of cultivated *Brassica rapa* were 2.34-4.36 g, up to 7.7 g; the average TSWs of cultivated *Brassica juncea* were 1.66-5.04 g, up to 8.0 g and the average TSWs of rapeseed were 2.0-4.0 g. Chinese rapeseed germplasms were mainly introduced from European countries. The TSWs of currently cultivated rapeseed varieties ranges from 3.0 g to 3.5 g, indicating the scarity of large seed rapeseed germplasms compared with *Brassica rapa* and *Brassica juncea*. Therefore, it is necessary to create new rapeseed germplasms with good economic traits, especially high TSW and oil content.

Here, creation of an extra-large seed rapeseed germplasm GM01 with the TSW of 8.68 g was reported. It was selected from the DH (Double Haploid) lines of a three-way cross: "020010"—a semi-winter late-maturing rapeseed variety, "Legency"—a spring Canadian variety and H8—a spring early-maturity Yunnan local rapeseed variety. Among these DH lines, 53 lines had TSW more than 5 g. In 2010-2011, GM01 was evaluated in four locations of Yunnan, China (Kunming, Chuxiong, Lijiang and Xiaoshao) and two locations of Hubei, China (Wuhan city and Ezhou).

2. Material and Methods

2.1 Experimental Procedures

In the February, 2002, a cross was made between a semi-winter late-maturing rapeseed germplasm "020010" (TSW of 4.6 g) and a spring Canadian variety "Legency" (TSW of 3.8 g). The F_1 seeds were harvested and planted in the June of 2002 and a cross was made between F1 of 020010/Legency and a Yunnan spring early-maturing rapeseed variety H8 (TSW of 3.6 g) (all the materials were deposited in the genetic stock of the Yunnan Academy of Agricultural Sciences). The F_1 of the cross 020010/Legency/H8 was planted in the October, 2002 and the buds of the F_1 plants were used to isolate microspores for culture. One hundred and forty eight DH lines were obtained and their TSWs were evaluated in 2004 and 2005. Among these DH lines, 69 lines had TSW of 4.0-5.0 g and 53 lines had TSW more than 5 g. Five lines with TSW higher than 7.0 g and H8 were evaluated in four locations mentioned above in the winter season of 2010-2014. The trials were set up in a Complete Randomized Block Design (RCBD) with three replications. Seeds had been handed planted in 2 × 2 m plots. Each plot contained 5 rows with 40 cm space between the rows and 16 plants per row with 15 cm space.

2.2 Crop Assessment

The morphological characteristics of GM01 and H8 were measured in the Kunming winter trial of 2010-2011. The length and width of petals, the length of stigmas and pistils, the diameter of pedicel, ovule number and the fresh weight of flower buds and ovaries were averaged from 50 fresh flower bud or flowers from the main stem at the beginning of flowering.

Agronomic traits including plant height, pods per plant, number of seeds per pod, TSW and yield per plant were averaged from ten randomly selected plants at maturity. The length and width of the silique were averaged from 50 siliques. The seed diameter was averaged from randomly selected 100 seeds from the 50 siliques. Seeds were air dried and were used in quality analysis with near-infrared FOSS analyzer.

Data were subjected to analysis of variance (ANOVA) or simple *t*-test using SPSS software.

3. Results

3.1 The Creation of GM01

In 2002, the three-way cross "020010/legency//H8" was obtained. In 2003, 148 DH plants were obtained from the isolated microspore culture of F_1 plants of "020010/legency//H8". These DH lines segregated in their TSW in the Kunming trail (Fig. 1). Among those DH lines, 53 lines had TSW over 5.0 g and the biggest TSW amounted to 8.68 g. According to TSW of the 2003 trial, 12 DH lines of high TSW (> 5.0 g) were selected for the winter trials of 2004 and 2005. The TSW of these DH lines were relatively stable between two years with variation of 10%-15% (Fig. 2).

Based on the field experiment of high TSW of GM01 line for multi-years and locations in different ecological environments, its TSW ranged from 6.2 g to 7.6 g and was significantly higher than the control H8, despite the impact of the various ecological environment of the planting years (Table 1).

3.2 Morphological Characteristics of GM01

3.2.1 Corolla Characteristics of GM01

Four petals of H8 and GM01 were all arranged in a typical cross figure. The petals of H8 were flat on the top whereas, those of GM01 were folded on the top. The four petals of H8 were largely separated from each other, whereas, those of GM01 were overlapped between two adjacent petals (Fig. 3). The lengths of the petals of GM01 and H8 were similar, but the widths of the petals of GM01 were about 30% broader than those of H8 (Table 2). The pedicel of GM01 was in the same length with that of H8, but it was about 78% thicker than that of H8. The lengths of flower bud were the same between GM01 and H8, and the diameter and fresh weight of flower buds of GM01 were higher than those of H8. The length of the ovary was the same between GM01 and H8 and they also

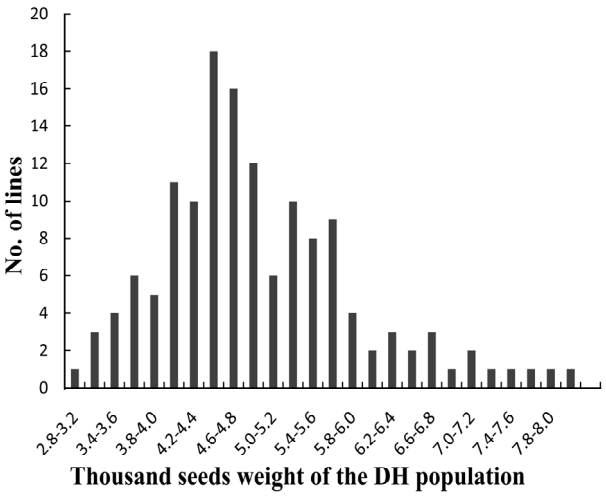

Fig. 1 TSW of 148 DH lines in the Kunming trial of 2003.

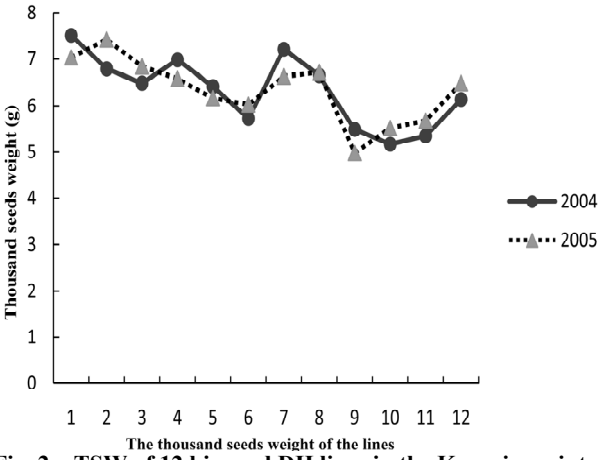

Fig. 2 TSW of 12 big seed DH lines in the Kunming winter trials of 2004 and 2005.

Table 1 TSW of GM01 in the trials of six locations in 2010-2014.

Location	Latitude	Altitude (m)	TSW (g)			
			2010	2011	2013	2014
Kunming	N25°E102°	1,910	7.15 ± 0.45**	7.63 ± 0.51**	7.02 ± 0.55**	7.09 ± 0.22**
Xiaoshao	N25°E103°	1,960	6.90 ± 0.57**	7.33 ± 0.77**	7.22 ± 0.41**	6.89 ± 0.43**
Cuxiong	N25°E102°	1,870	6.77 ± 0.37**	6.92 ± 0.57**	7.01 ± 0.61**	7.13 ± 0.31**
Lijiang	N26°E110°	2,400	6.88 ± 0.41**	7.12 ± 0.67**	6.79 ± 0.34**	—
Wuhan	N30°E112°	27	7.03 ± 0.38**	7.11 ± 0.56**	7.21 ± 0.45**	6.27 ± 0.47**
Ezhou	N30°E114°	20	7.03 ± 0.33**	7.07 ± 0.25**	7.21 ± 0.35**	—

* and ** refer to the significance of differences at the 0.05 and 0.01 level respectively.

Fig. 3 The flowers, siliques, stigma and seeds of GM01 and H8 (A: extra-large seed material "GM01"; A1: flower bud; A2: ovary; A3: flower; A4: petal; A5: pods; A6: stigma under scanning electron microscope (× 80); B: average seed material "H8"; B1: flower bud; B2: ovary; B3: flower; B4: petal; B5: pods and B6: stigma under scanning electron microscope (× 100)).

Table 2 The flower organ characteristics of GM01 and H8 in Kunming trial of 2010.

Flower organ	Measurements	GM01	H8	Lengency
Petal	Length (cm)	1.25 ± 0.22	1.24 ± 0.09	1.33 ± 0.11
	Width (cm)	0.96 ± 0.02**	0.66 ± 0.07	0.57 ± 0.04
Pedicel	Diameter (mm)	0.82 ± 0.08**	0.46 ± 0.07	0.41 ± 0.07
Flower bud	Length (cm)	7.51 ± 0.17	7.13 ± 0.38	0.69 ± 0.12
	Diameter (cm)	4.07 ± 0.17**	3.4 ± 0.12	3.3 ± 0.17
	Fresh weight (g)	49.05 ± 2.32**	35.14 ± 2.64	32.33 ± 1.66
Ovary	Length (cm)	0.82 ± 0.02	0.79 ± 0.05	0.77 ± 0.07
	Fresh weight (g)	7.73 ± 0.59**	5.53 ± 0.53	5.69 ± 0.47
	Ovule	30.33 ± 1.97	31.01 ± 1.33	31.41 ± 1.13
Stamen	Length (cm)	0.65 ± 0.065	0.68 ± 0.042	0.62 ± 0.033
Pistil	Length (cm)	0.71 ± 0.021**	0.67 ± 0.013	0.64 ± 0.021
Silique	Length (cm)	12.11 ± 0.86**	6.33 ± 0.77	6.71 ± 0.83
	Width (mm)	5.98 ± 0.49**	4.87 ± 0.36	4.41 ± 0.25
	Diameter (mm)	2.62 ± 0.74**	1.42 ± 0.44	1.29 ± 0.39
Seed	Fresh weight (g/1,000 seeds)	7.20 ± 1.13 **	3.47 ± 0.87	3.79 ± 0.77
	Dry weight (g/1,000 seeds)	7.08 ± 0.87**	3.21 ± 0.73	3.31 ± 0.67

* and ** refer to the significance of differences at the 0.05 and 0.01 level respectively.

Fig. 4 Stigma exsertion phenomenon of GM01.

bared the similar number of ovules, but the fresh weight of GM01 ovary was about 40% higher than that of H8 (Table 2).

3.2.2 The Pistil and Stigma of GM01

The pistil of GM01 grew quicker than its stamens and the stigma was longer than stamens, thus, the stigma projected from the stamens. Then, about 30% GM01 plants had stigma exsertion phenomenon, especially in summer (Fig. 4). Whereas, the pistil of H8 was equal to or less than its stamens and the stigma was parallel or below the anthers. Comparing

the tip of stigma of GM01 and H8, H8 had more mastoid cells at the tip of stigma than GM01, therefore, the mastoid cells appeared to be hemispherical hat over the style of H8 while mastoid cells only covered the top of the style of GM01 (Fig. 3A6).

3.2.3 The Siliques and Seeds of GM01

GM01 had characteristics of long silique, thick silique skin and big seed. The silique length, width and seed diameter of GM01 were significantly greater than those of H8 (Table 3, Fig. 3A5). The fresh weight and dry weight of GM01 siliques were three times of those of H8. The dry weight of seed and embryo were 2.5 times of those of H8 (Table 3).

3.3 The Main Agronomic Traits of Big Seed Germplasms

Five big seed DH lines including GM01, GM02, GM03, GM04 and GM05 were selected for two year. The days to maturity of these DH lines were all more than 185 days, much longer than 182 days for H8. Comparing to H8, these five big seed DH lines had mainly primary branches, but no or few secondary branches. They had less siliques per plant and seeds per silique. However, because of much higher TSWs of these five DH lines, their yield per plant was significantly higher than that of H8 (Table 4).

3.4 Quality Traits of Five DH Lines and H8

The seed oil contents of GM01, GM02, GM03, GM04 and GM05 ranged from 38.18% to 42.13% with little advantage over that of H8. Their seed protein contents ranged from 23.95% to 25.94%, which were higher about 0.3%-2% than that of H8. However, the glucosinolate and erucic acid contents of these DH lines were significantly higher than that of H8 (Table 5).

4. Discussion

TSW is a complex quantitative traits controlled by the main genes, plus multiple minor genes, and easily affected by environment [15, 16]. The probability of obtaining big seed lines through conventional hybridization breeding is only 1/1,000 to 1/30,000, thus, it is difficult to obtain heritable large seed materials.

Isolated microspore culture is a cell (haploid) culture-based approach for producing completely homozygous DH plants from immature pollen grains

Table 4 Main agronomic traits of new germplasm.

Traits	Lines					
	GM01	GM02	GM03	GM04	GM05	H8 (CK)
Days of growth duration (days)	201	186	191	185	195	182
Height of plant (cm)	151.7 ± 5.17	148.8 ± 4.96	150.5 ± 2.17	152.7 ± 2.41	163.3 ± 3.14	157.3 ± 1.12
Diameter of main stem (mm)	19.55 ± 0.34	17.66 ± 1.09	16.24 ± 0.89	16.21 ± 0.66	16.51 ± 1.02	15.31 ± 0.52
No. of branch	8.7 ± 1.31	10.9 ± 0.96	7.4 ± 1.12	8.3 ± 0.33	11.1 ± 0.73	11.8 ± 0.24
Pods per plant	275.3 ± 15.42	262.6 ± 20.69	278.7 ± 11.13	206.2 ± 26.13	295.4 ± 11.35	357.4 ± 10.09
Seeds per pod	17.2 ± 0.33	18.2 ± 0.56	15.9 ± 0.27	19.4 ± 0.53	17.8 ± 1.02	21.6 ± 0.24
1,000 seed weight (g)	7.22 ± 1.17	7.12 ± 1.31	7.03 ± 0.59	6.78 ± 0.6	6.33 ± 0.65	3.37 ± 0.42
Diameter of seed (mm)	3.24 ± 0.06	3.21 ± 0.13	3.17 ± 0.07	3.07 ± 0.35	3.23 ± 0.14	1.87 ± 0.03

Table 5 The component analysis of seeds of DH lines and H8.

Lines	Oil content (%)	Protein content (%)	Glucosinolate content (μmol)	Erucic acid (%)
GM01	38.18 ± 0.58	25.8 ± 0.76	139.82 ± 15.51	33.89 ± 3.93
GM02	38.31 ± 0.71	25.94 ± 0.83	132.29 ± 13.27	31.24 ± 5.86
GM03	41.66 ± 0.73	23.73 ± 1.02	93.25 ± 11.13	25.11 ± 2.14
GM04	42.13 ± 0.51	23.95 ± 0.77	89.52 ± 5.93	24.17 ± 5.71
GM05	38.76 ± 0.81	25.1 ± 0.93	96.06 ± 7.53	23.31 ± 4.05
H8 (CK)	39.55 ± 0.73	23.48 ± 0.23	17.62 ± 1.06	1.11 ± 0.57

in a single generation [17, 18]. This technology is widely used in rapeseed breeding and its genetic analysis [19, 20] and has been adopted in many laboratories [21-24].

In this study, one out of 148 DH plants had TSW over 7 grams and other two DH plants had average TSW more than 7 grams in multi-location trials in 2004 and 2005 (Fig. 2). The probability of obtaining plants with TSW over 7.0 g was from 1/148 to 3/148, which was significantly higher than that of the conventional breeding.

The newly created DH lines (GM01, GM02, GM03, GM04 and GM05) not only produced big seed with TSW of 7.0 g in high altitude regions such as Kunming, Chuxiong and Lijiang of Yunan province, but also in low altitude plain of Wuhan and Ezhou of Hubei province. This indicates that there are major genes controlling the big seed characteristic in these DH lines.

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