High-purity Seed Production of Doubled Haploid Chinese Cabbage [*Brassica rapa* L. ssp. *pekinensis* (Lour.)] Through Microspore Culture

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ABSTRACT The purpose of this study was to produce doubled haploid red color heading type Chinese cabbage using isolated microspore culture. Genotypic differences in embryogenic response and regenerative ability of microspore-derived embryos to plants were observed. A high rate of plant regeneration from microspore-derived embryos was achieved by an improved protocol involving replacement of culture media and adjustment of heat shock temperature. More than 60% of regenerated plants were spontaneous doubled haploids. Haploids were characterized by short and malformed stamen and few viable pollen grains as compared to spontaneous doubled haploids and dihaploids. The seeds harvested from the 142 spontaneous doubled haploid plants were designated as H1; 25 seeds in each population was grown and characterized. The H1 generation has been shown to display considerable phenotypic variation and high purity. These spontaneous doubled haploids may be directly exploited as new varieties in a red colored Chinese cabbage breeding program.

Keywords Double haploid, Microspore culture, Plant regeneration, Red color heading type Chinese cabbage, Seed

INTRODUCTION

Red color heading type Chinese cabbage [*Brassica rapa* L. ssp. *pekinensis* (Lour.)] is an important crop receiving greater attention by seed companies due to the increased market demand. Most of the commercial cultivars of Chinese cabbage are F₁ hybrids, which require a sufficient degree of homozygous parental lines. Conventional breeding in Chinese cabbage is time-consuming and labor-intensive. Haploid breeding is more efficient than conventional plant breeding for the generation of diploid homozygous lines. Generally, to obtain a pure homozygous line from a hybrid, it needs at least six or seven generations by self fertilization, while it only needs a single or several generations by haploid breeding.

Microspore culture potentially offers a very effective means of producing homozygous lines of *Brassica* plants. Since the successful isolation of microspore culture of *Brassica napus* was reported (Lichter 1982), microspore

culture has been reported in other *Brassica* species (Sato *et al.* 1989; Baillie *et al.* 1992; Cao *et al.* 1994; Wong *et al.* 1996; Wang *et al.* 2009). However, microspore culture was not very successful in *B. rapa* (Guo and Pulli 1996), compared with *B. napus* (Zhou *et al.* 2002; Gu *et al.* 2004; Leroux *et al.* 2009). Just as there are differences in embryogenic response among species, there are also genotypic differences in microspore culture response. Thus, the routine protocols of microspore culture of *Brassica* cannot be simply used for a broad spectrum of breeding materials and should be clarified many factors such culture media and culture conditions for novel materials.

Nowadays doubled haploidy is being featured in cultivar production of many crops. According to Thomas *et al.* (2003), there are currently around 300 doubled haploid derived cutivars in 12 species, but this is likely to be a gross underestimate, as information from some countries is scant and many breeders are reluctant to reveal their production

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methods. Doubled haploidy has been extremely effective in cultivar production in barley, rice and rapeseed. The barley cultivar “Mingo” was the first major double haploid crop cultivar and produced and grown in Canada in the late 1970’s (Ho and Jones 1980). The blackleg resistance rapeseed doubled haploid cultivar “Quantum” captured 30% of the acreage in Canada in 1995 (Stringham *et al.* 1995).

Aiming to improve the embryoid induction rate and produce haploid plants for *Brassica* breeding, in this paper, we study the effects of genotypes, temperature preculture, and culture medium, on the microspore embryo formation.

MATERIALS AND METHODS

Plant material and growth conditions

A set containing five genotypes of *Brassica rapa* L. ssp. *pekinensis* (Lour.) (AS20, 21, 23, 24, 34) was tested as donor plants. The donor plants were grown in a growth chamber at 23/20°C (day/night) with a 16/8 h light/dark photoperiod until they had eight leaves. Vernalization of donor plants was conducted with a combination of 8°C and 8 h photoperiod for eight weeks. They were then grown to flowering in a growth chamber at 23/20°C (day/night) with a 16/8 h light/dark.

Isolation of microspores

The microspore isolation procedure was based on the study by Sato *et al.* (1989) with some modifications. Unopened young flower buds 2.0 to 3.0 mm in length were sterilized in sodium hypochlorite solution containing 2% for 10 min. The buds were washed three times with sterilized distilled water for 5 min, and then gently macerated in a washing solution B5 medium (Gambory *et al.* 1968) supplemented with 13% sucrose for 5 min using mortar and pestle. The macerated buds were passed through sieves (45 μ m), the filtrates were centrifuged three times at 1000 rpm for 3 min and the supernatants were discarded. The pellets were resuspended in culture medium. The harvested microspores adjusted to a density of 2×10^5 /ml in culture medium were plated in 60 $\times$ 15 mm plastic petri-dishes containing 2.5 ml liquid medium. An addition of a 0.1 ml drop of 1% activated charcoal to the culture dishes was plated. The dishes were sealed with parafilm.

Pretreatments and culture of microspores

In the heat shock experiment, isolated microspores were incubated under dark conditions at 30 and 32.5°C for 1, 2 and 3 days (Table 1), and then maintained at 25°C under dark conditions for 17 days. To test the effect of culture medium on embryogenesis, the NLM medium and half-strength NLM medium supplemented with different

Table 1. Treatments of the heat shock and culture medium of red colored Chinese cabbage in this study.

B. N.	Culture medium	Temperature condition	
		Temperature(°C)	Time(d)
AS20	1, 2, 3, 4	30, 32.5	1d, 2d, 3d
AS21	1, 2, 3, 4	30, 32.5	1d, 2d, 3d
AS23	1, 2	30, 32.5	1d, 2d, 3d
AS24	1, 2, 3, 4	30, 32.5	1d, 2d, 3d
AS34	1, 2, 3, 4	30, 32.5	1d, 2d, 3d

Table 2. Culture medium for microspore culture of red colored Chinese cabbage in this study.

No.	Culture medium
1	NLN + S13% + CaNO ₃ 0.5 g/L + NAA0.5 mg/L + BAP0.5 mg/L
2	NLN + S13% + CaNO ₃ 0.5 g/L + NAA1.0 mg/L + BAP1.0 mg/L
3	NLN + S15% + CaNO ₃ 0.5 g/L + NAA1.0 mg/L + BAP1.0 mg/L
4	1/2NLN + S13% + CaNO ₃ 0.25 g/L + NAA0.5 mg/L + BAP0.5 mg/L

concentration of growth regulators were tested for their efficient embryogenesis (Table 2). Various concentrations (0.5 and 1.0 mg/L) of 6-benzylaminopurine (BAP) and naphthalene acetic acid (NAA) were tested. Each treatment was carried out with three independent replications (three cultures).

Plant regeneration and soil transformation

After 17 days of microspore culture, the dishes were transferred to a slow rotary shaker (80 rpm) under a photoperiod of 16 h at 23°C until the embryos became green. At the cotyledon stage, embryos were transferred to dishes containing solid MS (Murashige and Skoog 1962) medium. Approximately three weeks later when shoots

developed from the embryos, they were transferred onto solid 1/2MS medium for rooting. The conversion percentages of microspore embryos to plantlets were calculated. After four weeks, rooted plantlets were vernalized at 8°C for at least two months. Thereafter they were transferred to a soil-perlite mixture in a pot and were kept for two more weeks at 23°C, with low light intensity, and high humidity. This was followed by gradual adaptation to glasshouse conditions.

Determination of doubled haploid

The proportion of spontaneous doubled haploid was calculated by dividing the number of fertilized plants by the total number of plants observed.

Fig. 1. Effect of heat shock temperature and duration on microspore embryogenesis in five genotypes.

Fig. 2. Effect of growth medium on microspore embryogenesis in five genotypes. Four different media (1, 2, 3, 4) were used for microspore culture (Table 2).

Doubled haploid (DH) line generation

Flower morphology and fertility of plants were investigated. Doubled haploid plants were grown and allowed to self-pollination to produce the corresponding seed (H1). For investigation of homozygous purity of H1, seeds were sown in the experimental field.

RESULTS

Effect of temperature and duration of heat shock

In the current study, five F₁ hybrids of Red color heading type Chinese cabbage were evaluated for their capacity to produce microspore-derived embryos on various conditions. Five genotypes were successfully induced to produce embryoids. Evident differences in microspore embryogenesis were observed among the five tested donor plants (Table 3). The effect of different temperature and duration of heat

shock prior to culture at the normal temperature (25°C) on the embryoid induction was investigated. For whole genotypes, the optimum heat shock treatment observed was at 30°C for one day which resulted in 62 ~ 604 embryoids; however, longer duration decreased the number of embryoids (Fig. 1).

Effect of culture medium on microspore embryogenesis

The effect of the concentration of NLM medium on isolated microspore culture was studied. The results were presented in Fig. 2. It showed that the embryoid induction was significantly increased with full-strength NLM medium. The combination of 0.5 mg/L BAP and NAA was very promising; the embryo induction was increased up to two-fold on full-strength NLM, which is significantly higher than 1.0 mg/L BAP and NAA. It can be seen that concentration of NLM as well as growth regulators play an important role on the embryoid induction in isolated microspore culture of red colored Chinese cabbage.

Table 3. Effects of heat shock and the combinations of culture medium on embryoids induction.

B.N	Culture media	Heat treatment					
		30°C 1d	30°C 2d	30°C 3d	32.5°C 1d	32.5°C 2d	32.5°C 3d
AS20	1	427 ± 98	184 ± 100	42 ± 25	0 ± 0	0 ± 0	19 ± 5
	2	171 ± 59	56 ± 20	26 ± 12	0 ± 0	0 ± 0	23 ± 5
	3	6 ± 2	2 ± 1	0 ± 0	0 ± 0	0 ± 0	0 ± 0
	4	0 ± 0	0 ± 0	0 ± 0	31 ± 9	55 ± 19	0 ± 0
AS21	1	253 ± 58	175 ± 23	59 ± 13	0 ± 0	0 ± 0	45 ± 17
	2	2 ± 1	0 ± 0	25 ± 9	0 ± 0	0 ± 0	41 ± 9
	3	7 ± 3	31 ± 15	0 ± 0	0 ± 0	0 ± 0	0 ± 0
	4	0 ± 0	0 ± 0	0 ± 0	42 ± 12	41 ± 22	0 ± 0
AS23	1	39 ± 8	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
	2	23 ± 10	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
AS24	1	166 ± 42	96 ± 11	0 ± 0	0 ± 0	0 ± 0	0 ± 0
	2	19 ± 13	7 ± 4	0 ± 0	0 ± 0	0 ± 0	0 ± 0
	3	2 ± 1	37 ± 2	0 ± 0	120 ± 22	21 ± 9	0 ± 0
	4	3 ± 1	0	28 ± 19	39 ± 8	23 ± 11	0 ± 0
AS34	1	241 ± 33	245 ± 67	0 ± 0	0 ± 0	0 ± 0	0 ± 0
	2	72 ± 42	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
	3	38 ± 9	85 ± 45	0 ± 0	120 ± 55	125 ± 29	0 ± 0
	4	0 ± 0	0 ± 0	0 ± 0	12 ± 7	0 ± 0	0 ± 0

Plant regeneration and acclimatization

The embryos at the cotyledonary stage (21 days old) were planted onto hormone-free solid MS medium for germination and further development (Fig. 3A). After three weeks, some embryos rapidly developed into plants. These germinated embryos were transferred to plastic or glass vessels containing solid 1/2MS for rooting (Fig. 3B-C). The embryos that developed abnormally showed swollen hypocotyls and cotyledons. The percentage of plant regeneration varied from 16.2% (AS21) to 3.2% (AS23), as shown in Table 4. The plantlets were transferred to a cold frame to vernalize for eight weeks (Fig. 3C). After vernalization in culture bottle, plantlets were transferred to soil in a greenhouse (Fig. 3D). The percentage of planting

of plantlets was 80.3% (AS24) to 0% (AS23) (Table 4). The proportion of spontaneous doubled haploid was calculated by dividing the number of doubled haploid plants by the total number of plants observed. Different spontaneous doubling frequencies were observed for different genotypes (Table 4). However, difference of the fertility rate was not detected depending on medium. The maximum spontaneous doubling frequency was observed in AS24 (64.2%), followed by AS34 (45%).

Seed purity of doubled haploid population

After about two months, the regenerated plants blossomed. The differences between plants of haploid and doubled haploid were analyzed at the morphological level. Doubled

Table 4. Differences of embryoids and fertile plant production through isolated microspore culture of Red Chinese cabbage.

B.N	No. of culture media	Number of embryoid (A)	Plantlets		Planting		Fertility	
			Number (B)	B/A (%)	Number (E)	E/B (%)	Number (F)	F/E (%)
AS20	1	672±228	88	13.1	51	58.0	29	56.9
	2	276±96	36	13.0	17	47.2	9	52.9
	3	8±3	1	12.5	0	0	0	0
	4	86±28	11	12.8	4	36.4	2	50.0
	Total	1042±355	136	13.1	71	52.2	41	57.7
AS21	1	532±111	87	16.4	59	67.8	29	49.2
	2	68±19	8	11.8	4	50.0	2	50.0
	3	38±18	4	10.5	3	75.0	2	66.7
	4	83±34	18	21.7	11	61.1	7	63.6
	Total	721±182	117	16.2	77	65.8	40	51.9
AS23	1	39±8	2	5.1	0	0	0	0
	2	23±10	0	0.0	0	0	0	0
	Total	62±18	2	3.2	0	0	0	0
AS24	1	262±53	30	11.5	25	83.3	17	68.0
	2	26±17	2	7.7	2	100.0	1	50.0
	3	180±34	24	13.3	19	79.2	12	63.2
	4	93±39	10	10.8	7	70.0	4	57.1
	Total	561±143	66	11.8	53	80.3	34	64.2
AS34	1	486±100	51	10.5	38	74.5	16	42.1
	2	72±42	9	12.5	5	55.6	2	40.0
	3	368±130	28	7.6	17	60.7	9	52.9
	4	12±7	1	8.3	0	0	0	0
	Total	938	89	9.5	60	67.4	27	45.0

Fig. 3. Differentiation of haploid and spontaneous doubled haploid plants through isolated microspore culture of red colored Chinese cabbage. (A) Microspore-derived embryoids after three weeks of culture. (B) Plantlets from embryoids after four weeks. (C) Vernalization of plantlets 8°C for at least two months. (D) Acclimatization of vernalized plantlets. (E-F) Flower of spontaneous (E) doubled haploid and (F) haploid plants.

Fig. 4. Seed purity of doubled haploid population through isolated microspore culture of red colored Chinese cabbage. (A) Flowering shoots and silique of spontaneous doubled haploid (right) and diploid (left) of red colored Chinese cabbage. (B) Self-pollination of doubled haploid plants. (C) Field test of next generation (H1) of 142 spontaneous doubled haploid population. (D-E) H1 displayed variation for phenotype and uniformity in field.

haploid plants were fertile with normal anthers and many viable pollen grains (Fig. 3E). Haploid plants were sterile with short and malformed stamen and few viable pollen grains (Fig. 3F). The diameter of a flower and the length of anthers in haploids decreased by 1.4 and 1.8 times compared to doubled haploids, respectively (data not shown). Differences of flower and silique morphology were not observed between doubled haploid and dihaploid plants. Silique has pure seeds after self-pollination (Fig. 4A). The seed harvested from doubled haploid plants was designated (H1). A total of 25 seeds (H1) from each doubled haploid population were sown to observe uniformity in the field. The H1 generations displayed a large genotypic variation and were considered fixed (Fig. 4 C-E).

DISCUSSION

Microspore culture of *Brassica* crops is an attractive system for production of haploid plantlets. However, an efficient protocol for genotype-independent haploid induction is still lacking and the use of doubled haploids is limited. Genotype of the donor plant is regarded as one of the important factors affecting microspore culture in *Brassica* crops (Guo and Pulli 2000; Dias and Correia 2002; Zhang *et al.* 2008). Our experiment indicated that the best concentration of hormone for embryo yield was the combination of 0.5 mg/L BAP and NAA on full-strength NLM medium for whole genotypes. The heat shock treatment at 30°C for one day gave higher formation of embryo. The efficiency of microspore culture was different among five genotypes, and only three of five genotypes responded positively. It indicated that the different genotypes of red colored Chinese cabbage required different culture conditions. To increase the efficiency of microspore culture of red colored Chinese cabbage, culture medium and condition should be examined extensively.

In this study, plant regeneration frequencies from embryos are often low and variable (3.1~16.2%) depending on genotypes (Table 4). The average plant regeneration frequency was 10.2%, which is higher than those in other studies, while the efficiency of plant regeneration was 5~10% in red colored Chinese cabbage (Sato *et al.* 1989), and

7.6% in Pak Choi (Cao *et al.* 1994). However, not all embryos formed in microspores were able to regenerate into fully developed plants. Embryo quality and the frequency of conversion to plants are still a problem. The concentration of medium has been reported to affect the embryogenesis in microspore culture (Dias 1999; Wakui *et al.* 1994). Heat shock conditions and media osmolarities have shown to greatly affect abnormal embryo production in microspore culture (Hoekstra *et al.* 1993). The maintenance of osmolarity at a constant level is important for a high frequency of normal embryo induction and development.

For haploid microspore-derived plants to be useful in a breeding program, chromosome doubling to produce homozygous lines is necessary. In *Brassica*, the occurrence of spontaneous diploids is primarily governed by genetic factors and influenced by the microspore stage and the culture conditions (Keller and Armstrong 1978; Charne *et al.* 1988). A high percentage of spontaneous diploids were found in red colored Chinese cabbage (Zhang *et al.* 2001). Half of 45 genotypes produced 70% of spontaneous diploid plants. A high percentage of spontaneous diploids were found in broccoli (55%~71%) and cauliflower (56%) (Wang *et al.* 1999). Such high spontaneous diploidization has not been reported in other *Brassica* species. In *B. rapa* ssp. *oleifera*, the percentage of spontaneous diploids reported is 30%, and 5~50% in *B. napus* (Keller and Armstrong 1975; Charne *et al.* 1988; Chen and Beversdorf 1992; Mollers *et al.* 1994; Zhou *et al.* 2002a, 2002b). In the present study, the spontaneous doubling frequency was near 60% (Table 4), which is sufficient for exploitation in actual DH breeding programs. Thus an additional step of treatment is not required with chromosome doubling agents.

In this study, the differences between plants of haploid and doubled haploid plants at morphological levels in red colored Chinese cabbage (Fig. 3E-F). In *Brassica* crops, the difference between plants of different ploidy was also analyzed at the morphological level (Soroka 2013; Zhang *et al.* 2012). At the same time, pure seeds from doubled haploid plants were successfully obtained (Fig. 4). The pure seed production ability appeared to be similar in both parents. The H1 generations displayed significant variation for phenotype and were considered fixed (Fig. 4C-E). A

doubled haploid line is valuable for crop breeding programs because traits are fixed in just one generation, thereby reducing time and cost of cultivar development (Dunwell 2010; Ferrie and Caswe 2010; Germana 2011). Until now, we have obtained 142 doubled haploid plants in four genotypes of red colored Chinese cabbage. The results in the present study are useful for breeding programs in red colored Chinese cabbage.

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