Development and primary genetic analysis of a fertility-temperature-sensitive polima CMS restorer in *Brassica napus*

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Abstract

Over the past decade, the polima cytoplasmic male sterility (*pol* CMS) 'three -line' and 'two- line' systems have been developed for hybrid seed production in *Brassica napus* oilseed rape in China. We describe here the discovery of the novel *pol* CMS restorer line 'FL-204'. It restores male fertility of hybrid plants in the *pol* CMS system, but hybrid seed production can only be carried out under autumn sowing in Wuhan in south China under moderate temperatures at flowering. The restorer cannot be used as male for hybrid seed production in northwestern China (Gansu) under spring sowing conditions, where it is more or less male sterile due to high temperatures at flowering. Because of this behavior, it is referred to as fertility-temperature-sensitive-restorer(FTSR) in this paper. F_2, BC_1 as well as double haploid populations were constructed to determine the inheritance of fertility restoration of 'FL-204' under autumn at Wuhan and spring sowing condition at Gansu, respectively. Deviations from Mendelian genetics were observed. It was hypothesized that the change of fertility was the result of the interaction between nuclear genes (restoring gene *(Rf)* and temperature-sensitive genes (*ts*)) and the cytoplasm. The *Rf* gene in 'FL-204' was incapable of restoring male fertility of *pol* CMS lines under spring sowing conditions at Gansu where it is inactivated by the recessive *ts* gene present in 'FL-204'. However, the *ts* gene(s) could be nonfunctional under moderate temperature conditions at flowering at Wuhan which allows full expression of male fertility in 'FL-204'. The relationship between *ts* gene(s) and *Rf* can only be exhibited in plants containing the *pol* sterile cytoplasm. A method for the utilization of the FTSR *pol* CMS restorer 'FL-204' for hybrid seed production in *B.napus* oilseed rape is proposed.

Key words: *Brassica napus*, polima cytoplasmic male sterility, restorer, temperature-sensitive gene, fertility

Introduction

Hybrid vigor or heterosis is the superior performance of heterozygous hybrid progeny over both homozygous parents. Oilseed rape (*Brassica napus* L.) shows high levels of heterosis which the reason that oilseed rape breeders worldwide are interested in the development of F_1 hybrid cultivars. However, commercial production of hybrids requires a reliable and cost-effective pollination system for the production of hybrid seed. Different methods can be used to prevent self-pollination of the female line, such as application of male-specific gametocides, genetic male sterility and cytoplasmic male sterility. Cytoplasmic male-sterile (CMS) lines have a mutation in their mitochondrial genome, and the male sterility is inherited as a dominant, maternally transmitted trait. CMS can be used for hybrid seed production only if (1) CMS mutants are available; (2) (nuclear) restoring genes are available to restore the fertility of the CMS lines when seed is the commercial product; and (3) the CMS mutation is not associated with a yield penalty (Eva and Michiel 2002). In spite of these difficulties, for *B.napus*, CMS mutants are successfully used for hybrid seed production since spontaneous male sterile plants were found in Polima (*pol* CMS) in 1972 (Fu 1981, Fu et al.1995). *Pol* CMS lines can be divided into three categories according to the sensitivity of male sterility to temperature: high temperature CMS lines, low temperature CMS lines and stable CMS lines (Fu et al. 1990). For the stable *Pol* CMS lines, maintainer lines are essential for the multiplication of *pol* CMS lines while being used as a female parent in hybrid seed production. As far as temperature-sensitive *pol* CMS, although 'two-line' system (*pol* CMS and restorer lines) has been successfully used in China (Yang and Fu, 1990), the climate might be a problem in hybrid seed production. In practice, under 'abnormal' temperature condition, trace pollen can result in self-pollination within the CMS lines, which will lead to yield loss due to low hybridity levels. In some cases, the proportion of selfed seed was so high that the yield of the hybrid cultivar was significantly decreased in a large area (Liu and Yang 1992).

Besides the *pol* CMS line, an effective restorer line is very important for the production of hybrid seed. Fortunately, male fertility restoration controlled by a single Mendelian dominant gene is the simplest and easiest system for F_1 hybrid cultivar-breeding (Yang and Fu 1990, Jean et al. 1997).

Here, we report a novel *pol* CMS restorer that is fertile when sown in autumn in Wuhan (in the South of China) but sterile in summer in Gansu (in the northwest of China). It is named 'fertility-temperature-sensitive restorer' (FTSR). The objective of this study was to conduct a genetic analysis of the novel *pol* CMS restore line to determine genetic factor(s) underlying the temperature sensitive expression of male fertility /sterility of this restorer.

Materials and methods

Male sterile, maintainer and restorer lines of the *pol* hybrid seed production system used in this study are described in Table 1.The *pol* CMS restorer line '7-6' was fully male fertile under autumn sowing condition at Wuhan in southern China and was partially male sterile when cultivated during the summer months at Gansu in northwestern China. The male sterile or partially male sterile phenotype of the restorer '7-6' was observed for several years at Gansu but not at Wuhan (Yang and Fu 2001, patent ZL 01106599.0). In Gansu, '7-6' exhibited some of the typical *pol* CMS floral characteristics, such as shorter stamens bearing small conical anthers and smaller petals. The *pol* restorer 'L1815' in S-cytoplasm, possessing a male fertility restoration capability for the *pol* CMS system, was crossed with the temperature sensitive *pol* restorer '7-6' to produce F_1 plants. F_1 plants were used as donors to develop double haploid (DH) lines through microspore culture as described by Shi and Liu(1993). The DH population (63 lines) was sown in autumn at Wuhan and in spring in Gansu, respectively in 2003 and 2004, for evaluation of male sterility/fertility segregation. The DH line 'FL-204' was selected as a temperature sensitive restorer line for further experimentation.

Influence of temperature on fertility of 'FL-204': Oilseed rape flowers in March at Wuhan and in July at Gansu. The month mean temperature at flowering period kept 10°C and 20°C at Wuhan and Gansu, respectively in 2003 and 2004. As for the temperature in flowering period in Gansu being higher than that in Wuhan, the temperature was considered as the first factor influencing the fertility of 'FL-204'. Therefore, high temperature experiment was designed to testify the assumption. The plants of 'FL-204' were grown in the growth chamber at 25/15°C (day/night) in 2003 and 2004 respectively, with 12hr light length. Fertility was observed after plants flowered.

The genetic analysis of 'FL-204': The *pol* fertility temperature sensitive restorer (FTSR) 'FL-204' was crossed with two *pol* CMS lines, '195A-14' and '8110A' in Wuhan to determine whether the restorer gene was still present in the line. In order to test the cytoplasm type, reciprocal crosses were made between 'FL-204' and the *pol* CMS maintainer '6-571B'. 'FL-204' was also crossed with two *pol* CMS restorers 'Lun31' and 'Hui5148' as males. F_2 and BC_1 populations from the crosses were established to study the inheritance of male fertility of 'FL-204'. Two DH populations were also produced from F_1 plants of plants of these two crosses. Male fertility evaluations were conducted for all segregating generations, including the original DH population derived from the cross, as indicated 'L1815 × 7-6' in Table 2.

Fertility scoring: Male fertility of individual plants was evaluated in the two locations, Wuhan and Gansu. In Wuhan (a winter rapeseed planting area), materials were sowed in autumn and blossomed next spring. In Gansu (a summer rapeseed planting area), materials grew from late May to late September. Based on the level of the anther development, the male fertility of flowers was classified into seven categories according to Yang and Fu (1991).

Results

Selection for FTSR in DH population derived from cross between pol *restorers 'L1815 × 7-6'*

By microspore culture, 63 DH lines were produced from F_1 plants of the cross 'L1815 ×7-6'. In 2003, all genotypes were completely fertile and petals were normal at Wuhan. However, at Gansu eleven DH lines exhibited partial or complete male sterility. The same results were observed in 2004. For the eleven DH genotypes, different degrees of male sterility were observed (Last row in Table 4). One DH genotype, designated as 'FL-204', was selected because it had crinkled petals and a very limited amount of pollen compared to other DH lines when grown at Gansu.

Fertility of 'FL-204' treated with day/night temperatures of 25/15 ℃

In spring 2003 and 2004, 'FL-204' was fertile when it was grown in the field in Wuhan (Fig 1a). But for plants grown under a 25/15°C(day/night) temperature regime in the growth chamber, they were found to be male sterile as in the field in Gansu (Fig 1b). The buds and petals of 'FL-204' in the growth chamber were obviously smaller than those in the field in Wuhan. In fact, judging from the crinkled petals and short stamens as well as conical anthers, it was difficult to distinguish them from *pol* CMS plants.

The fertility restoring ability of 'FL-204'

Since the phenotype of the flower in 'FL-204' in Gansu (Fig 1b) was distinguished from that in Wuhan (Fig 1a), it was not sure whether 'FL-204' was a *pol* CMS or restorer. Therefore, two *pol* CMS lines, '8110A' and '195A-14', were test-crossed with 'FL-204', respectively. The F_1 plants were found fertile in Wuhan while partially fertile in Gansu (Table 3). And in Gansu, the pollen produced in F_1 plants was much more than that in '8110A' and '195A-14'. It could be assumed that the fertility be restored by the restoring gene in 'FL-204' when the F_1 plants were planted in Wuhan but partially restored when the F_1 plants were planted in Gansu. Therefore, 'FL-204' certainly carries the *pol* CMS restoring gene, and it is a FTSR.

The cytoplasm type of 'FL-204'

The *pol* CMS maintainer '6-571B' was reciprocally crossed with 'FL-204'. The F_1 of '6-571B×FL-204' was fertile in Wuhan and Gansu (Table 3). The petals in the F_1 plants were as large as those in '6-571B'. However, the reciprocal F_1 of 'FL-204×6-571B' was partially sterile in Gansu vs. completely fertile in Wuhan(Table 3). The difference of fertility between reciprocal crosses indicated that the FTSR contained *pol* cytoplasm (designated as 'S'). This conclusion would be further proved by observing the fertility of F_2 and BC_1 populations derived from the reciprocal crosses between 'FL-204 and '6-571B' in the next part.

Fertility segregation in F_1, F_2 and BC_1 populations

In order to study the inheritance of the temperature-sensitive (*ts*) gene(s), 'FL-204' was crossed with two *pol* CMS restorers ('Lun31' and 'Hui5148'). All F_1, F_2 and BC_1 populations were found completely fertile in Wuhan. But in Gansu, the situation was much complicated. No sterile plants were found in all F_1 populations (Table 3). For the F_2 populations, one completely sterile, 24 partially sterile and 283 fertile plants were observed in the cross of 'FL-204 × Hui5148', and 23 partially sterile and 182 fertile plants were observed in the cross of 'FL-204 × Lun31'. In BC_1 populations, when 'Lun31' or 'Hui5148' was used as the backcross parent, all offspring in BC_1 were completely fertile at both locations. But when F_1 plants were crossed with 'FL-204', a higher ratio partially sterile plants(46/98) for '(FL-204 × Lun31)× FL-204' and 65/103 for '(FL-204 × Hui5148) × FL-204') were found in BC_1 populations than in F_2 populations.

The segregating populations derived from the cross between 'FL-204' and '6-571B' were also investigated in Gansu. In the F_2 and BC_1 populations from '6-571B×FL-204', all progeny were completely fertile because of their normal cytoplasm. While in the F_2 and BC_1 populations from 'FL-204×6-571B', most plants were sterile or partially sterile and a small number was normal fertile (Table 4).

Except for the backcross population '(FL-204×Lun31)×FL-204', all the other segregation in the populations from the crosses either between 'FL-204' and restorers or between 'FL-204' and the maintainer was not in accordance with any Mendelian inheritance model.(Table 4 in which two genes, temperature-sensitive gene '*ts*' and restorer gene '*Rf*',were proposed to control the fertility and χ^2- test (P⩽0.05) was used to test the genetic models.) It is suggested that the inheritance of FTSR is quite complicated, but results indicate that (1) the *ts* gene(s) was not the allele of the *Rf* gene and (2) the *ts* gene(s) had quantitative effect on the *Rf* gene.

Fertility segregation in DH populations

For a better understanding of the inheritance of the FTSR, three DH populations were developed. They were from the combination of 'FL-204 × Hui5148', 'FL-204 × Lun31' and 'FL-204 × L1815', respectively. All DH populations were normal fertile in Wuhan, but segregation was observed in Gansu (Table 4,Fig 2). The fertile flowers were bigger than the sterile flower (Fig 2a and Fig 2b). Also all the fertility segregation ratios in the three DH populations did not fit any Mendelian genetic model, which was consistent with the results from the F_2 and BC_1 populations.

Discussion

By evaluation the fertility of 'FL-204' and its offspring in different locations, we demonstrated that the environment played an important role in the expression of male fertility of 'FL-204'. In fact, the effects of environmental conditions on cytoplasmic and genic male sterility have been investigated in many other crops, such as broccoli (Dickson 1970), soybean (Stelly and Palmer1980), common bean (Estrada and Mustschler1984) and rice (Li et al. 2002). Compared with the climate in Wuhan, the photoperiod is longer and the temperature is higher in Gansu in summer. The controlled temperature experiment indicated that high temperature could lead to male sterility in 'FL-204' at Gansu, but the critical temperature causing fertility conversion still needs to be determined.

Yang et al (1995) reported a promising male sterile line, 'ecotypical male fertile-sterile line AB_1'. 'AB_1' was partially fertile when it was sown at Wuhan in autumn, but became sterile when sown at Kunming or Xining in summer. The change of fertility was the result of interaction between the cytoplasm and the nuclear genes (temperature-sensitive genes). Yet 'AB_1' was a *pol* CMS line and did not fit the same genetic model as 'FL-204'. Firstly, 'AB_1'were partially fertile, but 'FL-204' was fully fertile when both were planted in autumn in Wuhan. Secondly, the F_1 of 'AB_1 × *pol* CMS maintainer' was partially sterile, but the F_1 of 'FL-204 × *pol* CMS maintainer' was fully fertile when both were planted in autumn in Wuhan. Thirdly, all plants were sterile in F_2 and BC_1 population in summer when 'AB_1' as female parent was crossed with *pol* CMS maintainers, but fertile offspring were found in F_2 and BC_1 population in summer when 'FL-204' as female parent was crossed with the *pol* CMS maintainer. Fourthly, fertility segregation of F_2 and BC_1 from crosses between 'AB_1' and *pol* CMS restorers fitted the Mendelian model of one major gene when they were planted in the Gansu, but those from crosses between 'FL-204' and *pol* CMS restorers did not. Based on these differences, it was assumed that 'FL-204' was another type of temperature sensitive material with a *pol* CMS restoring gene (*Rf*) instead of a sterile gene (*rf*), and there could be another gene or other genes affecting the action of the *Rf* genes.

It has been reported that the partially dominant *Ts* genes in 'AB_1' are multiple minor genes, which can play the role of restorer genes when 'AB_1' is planted in autumn in Wuhan, but they are inactivated when 'AB_1' is planted in summer in Gansu (Yang et al.1995). But the *ts* gene(s) in 'FL-204' are probably prohibiting or epistatic gene(s) which is inactive in Wuhan while they could be activated in Gansu so as to prohibit the *Rf* gene's capability of restoring the fertility of *pol* CMS. The *ts* gene(s) is certainly not the allelic gene of the *Rf*, because all F_1s between 'FL-204' and three *pol* CMS restorers were normal fertile and fertility segregations were observed in the F_2 and BC_1 populations when they were planted in Gansu in summer. The phenomenon suggested that there was another dominant allele of the *ts* gene(s).

Previous research found that Mendelian dominant mechanism controlled the inheritance of *B.napus pol* CMS restorer gene (Fang and McVetty1989). But here we describe a case of severe deviation from the Mendelian genetic model in F_2 and DH populations derived from 'FL-204× *pol* CMS restorers' in our experiment. It indicated that the *ts* gene(s) could be linked to the *Rf* gene.

In the northwest of China, oilseed rape is grown throughout the growing season from May to September. However, in the south of China, it is sown in September and harvested in May next year. So, as the female parent, the novel temperature sensitive material is very suitable for production of hybrid seed in the northwest which then can be released for planting in the south of Chinese market.

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Table 1: Male sterility/fertility of polima CMS, maintainer and restorer lines of *Brassica napus* oilseed rape, developed by the rapeseed laboratory of Huazhong Agricultural University, under autumn sowing conditions at Wuhan and spring sowing conditions at Gansu, China, for two years(2003 and 2004).

Lines	Cytoplasm and nuclear genotypes[3]	Male sterility/fertility	
		Wuhan	Gansu
195A-14 *pol* CMS	S(*rfrf*)	Sterile	Sterile
8110A *pol* CMS	S(*rfrf*)	Sterile	Sterile
6-571B *pol* maintainer	N(*rfrf*)	Fertile	Fertile
7-6 *pol* restorer[1]	S(*??RfRf*)	Fertile	Partially sterile
L1815 *pol* restorer	S(*RfRf*)	Fertile	Fertile
Lun31 *pol* restorer[2]	N(*RfRf*)	Fertile	Fertile
Hui5148 *pol* restorer[2]	S(*RfRf*)	Fertile	Fertile

1=restorer for hybrid cultivar 'Huaza No.13'.
2=double haploid lines.
3=cytoplasm: S=male sterile, N=normal male fertile, Rf= *pol* restorer.

Table 2: Evaluation of segregating generations for male sterility/fertility of two crosses between the *pol* FTSR 'FL-204' and *pol* restorers 'Lun31' and 'Hui5148', the reciprocal cross between the *pol* FTSR 'FL-204' and *pol* maintainer '6-571B', and the original cross between the *pol* restorers '1815' and '7-6' that generated the *pol* FTSR line 'FL-204', at Wuhan and Gansu, China, in 2003 and 2004.

F_1 Combinations	Segregating generations	Location of fertility scoring
L1815×7-6	DH	Wuhan, Gansu
FL-204×Lun31	DH, F_1, F_2, F_1×FL-204, F_1×Lun31	Wuhan, Gansu
FL-204×Hui5148	DH, F_1, F_2, F_1×FL-204, F_1×Hui5148	Wuhan, Gansu
6-571B×FL-204	F_1, F_2, F_1×FL-204, F_1×6-571B	Gansu
FL-204×6-571B	F_1, F_2, F_1×FL-204, F_1×6-571B	Gansu

Fig 1: Flower of pol restorer 'FL-204' (1a) completely fertile at Wuhan; (1b) sterile at Gansu.

Fig 2: Different fertility grade in the DH population derived from the cross between *pol* fertility-temperature-sensitive-restorer(FTSR) line 'FL-204' and the restorer line 'Hui5148' at Gansu, China, in 2004. Left: sterile; Middle: partially sterile; Right: Fertile

Table 3: Male fertility of F_1 plants from six crosses between the *pol* FTSR 'FL-204' with two *pol* CMS lines('195A-14' and '8110A'), the reciprocal cross with the *pol* maintainer '6-571B' and two *pol* restorer lines ('Lun 31' and 'Hui5148'), evaluated at Wuhan and Gansu, China, 2003 and 2004.

F_1 combination	Fertility grades(Fertility)*	
	In Wuhan	In Gansu
195A-14×FL-204	6(Fertile)	3(Partially sterile)
8110A×FL-204	6(Fertile)	3(partially sterile)
6-571B×FL-204	6(Fertile)	6(Fertile)
FL-204×6-571B	6(Fertile)	3(Partially sterile)
FL-204×Lun31	6(Fertile)	6(Fertile)
FL-204×Hui5148	6(Fertile)	6(Fertile)

*fertility categories: 0= male sterile, 6= male fertile.

Table 4: Male fertility/sterility segregation in DH, F_2 and BC_1 generations of crosses of the *pol* FTSR restorer line "FL-204' with *pol* restorers and the *pol* maintainer line '6-671B' under field conditions at Gansu, China, in 2004.

Populations	Total plants	plants with different fertility grades							F:S**	ER***	χ^2	*P*-value
		0	1	2	3	4	5	6				
(FL-204×Lun31)F_2	205	0	2	3	6	12	14	168	182:23	3:1	20.03	≤0.005
(FL-204×Hui5148)F_2	308	1	0	1	11	12	22	261	283:25	3:1	75.31	≤0.005
(FL-204×6-571B)F_2	231	43	59	30	18	17	23	41	64:167	9:7		≤0.005
(6-571B×FL-204)F_2	257							257	257:0		1.23	
(FL-204×Lun31)×FL-204	98	1	8	13	16	17	15	28	43:55	1:1		0.25-0.5
(FL-204×Lun31)×Lun31	256							256	256:0		6.56	
(FL-204×Hui5148)×FL-204	103	0	0	10	23	32	20	18	38:65	1:1		0.025-
(FL-204×Hui5148)×Hui5148	277							277	277:0		74.56	
(FL-204×6-571B)×FL-204	116	19	19	22	18	27	8	3	11:105	1:1	110.41	≤0.005
(FL-204×6-571B)×6-571B	170	61	15	19	22	37	6	10	16:154	1:1		≤0.005
(6-571B×FL-204)×6-571B	269							269	269:0			
(6-571B×FL-204)×FL-204	214							214	214:0		49.49	
(FL-204×Hui5148)DH	146	1	1	2	8	18	74	42	116:30	1:1	11.61	≤0.005
(FL-204×Lun31)DH	38	0	1	0	1	6	16	14	30:8	1:1	25.40	≤0.005
(L1815×7-6)DH*	63	1	1	3	2	4	28	24	52:11	1:1		≤0.005

* FL-204 was selected from the DH population.
**F:S is abbreviated for 'fertile/sterile plants'.
***ER is abbreviated for 'expected ratios'.