Introduction

The production of oilseed rape (*Brassica napus* L.) has increased rapidly over the past decade, not only due to a marked growth in cultivation area, but also because of the availability of high-yielding winter varieties, including open-pollinated and hybrid varieties. However, strong breeding selection for zero erucic acid and low glucosinolate content in seed has produced a narrow genetic base in these recently developed cultivars (Friedt and Snowdon 2009, Wu et al. 2014). One strategy for broadening the genetic base of oilseed rape germplasm is to exploit the diploid progenitor species of *Brassica napus*, namely *B. rapa* and *B. oleracea*. Both these species possess tremendous variability in morphology and agronomic characteristics and represent a valuable resource for the improvement of pathogen and pest resistance, tolerance to abiotic stresses and heterosis (Wu et al. 2014). The resynthesis of new *B. napus* from interspecific crosses between the original ancestor species is thus likely to increase genetic variation in this species. Methods for creating resynthesized (RS) oilseed rape have been known for many years (Kräling 1987), but the development of new biotechnologies, such as *in vitro* culture (including *in vitro* pollination, embryo rescue and *in vitro* androgenesis) and molecular techniques for the detection of valuable genotypes, has allowed broad and targeted exploitation of RS *B. napus* (Girke et al. 2012a, Rahman 2013).

Nevertheless, RS *B. napus* lines are generally not suitable for direct breeding of oilseed rape, because of low quality seed traits, such as low seed oil content, high erucic acid level in the oil and high glucosinolate content in seed meal, as well as other undesirable agronomic traits derived from one or both progenitors (Girke et al. 2012a, 2012b, Jesske

Development of new restorer lines for CMS ogura system with the use of resynthesized oilseed rape (*Brassica napus* L.)

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Resynthesized (RS) oilseed rape (*Brassica napus* L.) is potentially of great interest for hybrid breeding. However, a major problem with the direct use of RS *B. napus* is the quality of seed oil (high level of erucic acid) and seed meal (high glucosinolate content), which does not comply with double-low quality oilseed rape. Thus, additional developments are needed before RS *B. napus* can be introduced into breeding practice. In this study, RS oilseed rape was obtained through crosses between *B. rapa* ssp. *chinensis* var. *chinensis* and *B. oleracea* ssp. *acephala* var. *sabellica*. RS plant was then crossed with double-low (00) winter oilseed rape lines containing the *Rfo* gene for Ogura cytoplasmic male sterility (CMS ogu) system. Populations of doubled haploids (DH) were developed from these F1 hybrids using the microspore *in vitro* culture method. The seeds of semi-RS DH lines were analyzed for erucic acid and glucosinolate content. Among the populations of semi-RS DHs four 00-quality lines with the *Rfo* gene were selected. Using 344 AFLP markers to estimate genetic relatedness, we showed that the RS lines and semi-RS lines formed clusters that were clearly distinct from 96 winter oilseed rape parental lines of F1 hybrids.

Key Words: *Brassica napus*, resynthesis, semi-resynthesis, winter oilseed rape, hybrid breeding.

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Abbreviations: semi-RS, semi-resynthesized line (line obtained by crossing oilseed rape × RS); 00-quality or double low, genotypes with zero erucic acid and low seed glucosinolate content; *Rfo* gene, restorer fertility ogura gene; CMS ogura, cytoplasmic male sterility Ogura system
New restorer line CMS ogura with resynthesized oilseed rape

et al. 2013). Poor seed sets in newly RS B. napus are a very common phenomenon, probably due to the unstable meiotic behavior of the parental genomes. The disturbances in fertility observed in RS oilseed rape result in a low number of seed sets and consequently in rather low seed yield (Girke et al. 2012b, Rahman 2005). However, some important positive effects on seed yield, including improved seed quality and other traits from the introgression of alleles from RS B. napus into genotypes of oilseed rape, have been observed. RS oilseed rape has previously been introduced into cultivar oilseed rape as a source of yellow seed color (Chen and Heneen 1990, Rahman 2001), earliness (Akbar 1989), club-root resistance (Diederichsen and Sacristan 1996) and self-incompatibility in the context of a control mechanism for hybrid seed production (Rahman 2005), for example. Seyis et al. (2006) reported that higher seed yield in hybrids, compared with open-pollinated varieties, can be achieved by the use of RS oilseed rape. Kräling (1987) investigated the use of RS oilseed rape to improve the seed yield of winter hybrids by crosses of cultivar × RS line and cultivar × (cultivar × RS). Other workers observed a beneficial effect on seed yield from the introgression of RS B. napus into spring hybrid combinations (Udall et al. 2004). Many other examples of the introduction of RS genotype oilseed rape into initial breeding material can be found in the literature (Girke et al. 2012a, 2012b, Seyis 2013, Wu et al. 2014).

The potential for hybrid cultivars in oilseed rape is very well documented (Seyis et al. 2006) and different systems for controlling cross pollination have been developed. One of the most widely used by breeding companies is the Ogura cytoplasmic male sterility (CMS ogura) system which is characterized by a sterile male cytoplasm and the Rfo restorer gene from Raphanus sativus L. (Renard et al. 1997).

The availability of genetically distant plant material is very important for hybrid breeding (Brandle and McVetty 1990, Lefort-Buson et al. 1987). However, the narrow genetic variation of oilseed rape limits the opportunity for hybrid breeding, since the optimal utilization of heterosis requires complementary gene pools. The use of artificially resynthesized oilseed rape offers the possibility of increasing genetic diversity and thereby developing genetic distance between heterotic pools for hybrid breeding. Since direct use of genetically diverse germplasm in breeding can also introduce unwanted traits/alleles from RS parental lines, the introgression of RS oilseed rape would require further cycles of breeding for improvement (Rahman 2013). The use of potential RS lines is therefore more appropriate for the development of semi-RS oilseed rape or backcrossing for the introgression of genetic diversity (Seyis et al. 2006, Seyis 2013), as well as for improving the quality of the novel genotypes. In this study, double-low (00) lines of winter oilseed rape carrying the Rfo gene were crossed with two RS B. napus lines. Two F₁ hybrids were used for the production of androgenic plant populations, which were analyzed both for the Rfo gene and also biochemically for double-low quality in their seeds.

The main objective of this study was to obtain 00-quality semi-RS DH lines of winter oilseed rape with Rfo gene which are genetically distant from the current, natural B. napus genotypes.

Materials and Methods

Plant materials

Resynthesized oilseed rape plants were obtained by crossing Brassica rapa ssp. chinensis var. chinensis (pak choy) and Brassica oleracea ssp. acephala var. sabellica (curly kale) using the embryo rescue technique, thereby doubling the chromsome number of regenerated plants (Sosnowska et al. 2010, Sosnowska and Cegielska-Taras 2014). RS2 plant was used as pollinators to cross with two double-low restorer lines of winter oilseed rape: 1202/3 and 1215/4. From two F₁ hybrids, 1202/3 × RS2 = 12r and 1215/4 × RS2 = 17r populations of androgenic plants were obtained, using isolated microspore culture in vitro (Cegielska-Taras et al. 2002).

Isozyme marker PGI-2

Androgenic plants were analyzed for the presence of the restorer Rfo gene by testing for the linked isozyme marker, phosphoglucoisomerase (PGI-2), (Delourme and Eber 1992, Popławska et al. 2007). For further study, only androgenic plants with the restorer gene were used.

Fatty acid composition and glucosinolate content

Seeds from semi-RS doubled haploid plants with the Rfo gene were analyzed for fatty acid composition and glucosinolate content using gas chromatography.

Molecular marker RAPD OPC021150 analysis

In the double-low quality semi-RS DH lines selected, the presence of the restorer Rfo gene was additionally confirmed by testing for RAPD OPC021150, a molecular marker of Rfo gene (Delourme et al. 1994).

Genetic similarity

The genetic similarity of the tested genotypes, including two RS B. napus lines, three semi-RS DH lines and 96 parental lines of winter oilseed rape F₁ hybrids, was determined by AFLP-PCR using 10 fluorescently labeled primer combinations (Sobotka et al. 2004).

These 96 parental lines of winter oilseed rape F₁ hybrids derived from Plant Breeding Strzelec Ltd., Co., Poland. Amplification products were analyzed by capillary electrophoresis (Applied Biosystems). The data matrix for all study genotypes and primer combinations, excluding monomorphic peaks, was used to calculate pairwise genetic similarity based on the coefficient of Nei and Li (1979). All calculations were performed using GenAlEx 6.5 in Excel and STATISTICA v.10 software (Peakall and Smouse 2012, StatSoft, Inc., Tulsa, USA).
**Analysis of principal components**

On the basis of the 344 AFLP markers, principal component analysis (PCA) was performed by GenStat v.17 statistical package. Principal components analysis explains the considerable genetic variation associated with the origin of the RS line, semi-RS lines and six natural cultivars of oilseed rape: cv. Jantar, cv. Bazyl, cv. Mohican, cv. Mendel, cv. Exocet, cv. Dante.

**Field experiment**

The field trials at two locations (Borowo and Łagiewniki, Poland) were arranged in a randomized complete block design, with four replicates in crop season 2014/2015. Each genotype was grown in a four-row plot of 10.0 m² (Borowo), and 9.6 m² (Łagiewniki) with a 0.30-row distance and a sowing density of 80 seeds/m². Plots were harvested using a plot harvester. Total grain yield was measured in dt per hectare (dt·ha⁻¹).

**Results**

The genetically distant species *B. rapa* ssp. *chinensis* (pak choy) and *B. oleracea* ssp. *acephala* var. *sabellica* (curly kale) were chosen for resynthesis of oilseed rape. Preliminary molecular analysis using RAPD markers showed a clear distinction between the RS lines obtained and the oilseed rape varieties and lines that are currently in breeding and cultivation (Sosnowska et al. 2010). RS plant (RS2) was chosen as pollinator line to cross with the double-low quality restorer DH lines 1202/3 and 1215/4. From two semi-RS F1 hybrids, denoted 12r and 17r, 801 androgenic plants were developed using isolated microspore culture (Table 1).

In the Ogura CMS system, the restorer gene *Rfo* is tightly linked to the co-dominant isozyme marker, phosphoglucoisomerase (PGI-2), (Delourme and Eber 1992). This marker has been effectively used to screen for the presence of *Rfo* gene alleles. (A) genotypes with *Rfo* alleles; (B) genotypes with *rfo* alleles.

After vernalization, 180 semi-RS doubled haploids with restorer gene were developed and the plants were grown in a greenhouse. Only a proportion of these plants formed proper seeds, but those seeds that did form were analyzed for fatty acid and glucosinolate content. Erucic acid level in the seed oil of 12r population ranged from 0 to 58.3% and from 0 to 51.2% in the 17r population. The calculated coefficients of variation were 51.92% and 54.15%, respectively (Table 2). Many of the semi-RS DH lines from both 12r and 17r populations were characterized by high level of erucic acid in the range from 2% to more than 40% (Fig. 2). Total glucosinolate content in seeds of DH lines of the 12r population ranged from 4.0 μmol g⁻¹ of seeds to 116.1 μmol g⁻¹ of seeds and in the 17r population from 3.1 μmol g⁻¹ of seeds to 117.5 μmol g⁻¹ of seeds in all analyzed genotypes. The coefficients of variation were 72.02% and 60.77%.

**Fig. 1.** Isozyme analysis for PGI-2 in androgenic plants obtained from F₁ hybrid 12r (restorer line *RfoRfo* × RS2) to indicate the presence of *Rfo* gene alleles. (A) genotypes with *Rfo* alleles; (B) genotypes with *rfo* alleles.

| F₁ hybrids | Minimum | Maximum | Mean | Standard deviation | Coefficient of variation (%) |
|------------|---------|---------|------|--------------------|-----------------------------|
| 1202/3 × RS 2 = 12r | 0.0 | 58.3 | 31.09 | 16.84 | 54.15 |
| 1215/4 × RS 2 = 17r | 0.0 | 51.2 | 27.84 | 14.45 | 51.92 |

**Fig. 2.** Distribution of DH lines with the *Rfo* gene according to erucic acid content.

| F₁ hybrids | Minimum | Maximum | Mean | Standard deviation | Coefficient of variation (%) |
|------------|---------|---------|------|--------------------|-----------------------------|
| 1202/3 × RS 2 = 12r | 4.0 | 116.1 | 43.87 | 31.60 | 72.02 |
| 1215/4 × RS 2 = 17r | 3.1 | 117.5 | 53.25 | 32.36 | 60.77 |

**Table 1.** Androgenic plants and plants with the *Rfo* gene from two F₁ hybrids obtained from crossing DH restorer lines × RS lines of oilseed rape

| F₁ hybrids | Number of androgenic plants obtained with *Rfo* gene |
|------------|--------------------------------------------------|
| 1202/3 × RS 2 = 12r | 406 | 120 |
| 1215/4 × RS 2 = 17r | 395 | 161 |
| Total | 801 | 281 |
New restorer line CMS \textit{ogura} with resynthesized oilseed rape

respectively (Table 2). The majority of semi-RS doubled haploids were characterized by more than 15 \( \mu \text{mol g}^{-1} \) of glucosinolate content in seeds (Fig. 3).

Finally, from all semi-RS doubled haploids studied, we selected four 00-quality lines (S1, S2, S3 and S4) with the \( \text{Rfo} \) gene (Table 3). The molecular marker RAPD OPC0215 confirmed the presence of the \( \text{Rfo} \) gene in these four semi-RS DH lines (Fig. 4). DH line S3 was developed from \( \text{F}_1 \) hybrid 12r and DH lines S1, S2 and S4 from \( \text{F}_1 \) hybrid 17r. The seeds of semi-RS DH lines S1, S2 and S3 were characterized by zero erucic acid, while S4 had trace level. The glucosinolate content in semi-RS DH line S4 was 4.4 \( \mu \text{mol g}^{-1} \), but in the remaining three lines it was less than 15 \( \mu \text{mol g}^{-1} \) seeds. Genetic similarity analysis by AFLP-PCR using 10 primer combinations generated 344 markers; these were used to characterize the two RS lines, three semi-RS lines and 96 parental lines of winter oilseed rape \( \text{F}_1 \) hybrids included in this study. The dendrogram (Fig. 5) shows several clusters for the 96 natural \textit{B. napus} strains and clearly distinct clusters of the RS lines R33/13 and R34/13, and the semi-RS DH lines R12 125/13 and S1, S2.

Principal components analysis performed on the basis of 344 AFLP markers clearly explains the considerable genetic variation associated with the origin of the RS line (RS 33/13 and RS 34/13), semi-RS restorer lines (S1, S2 and R12 125/13) and six cultivars of winter oilseed rape (Fig. 6).

The first results obtained from the field experiment carried out in two environments with the two \( \text{F}_1 \) hybrids obtained from the same maternal line – CMS \textit{ogura} (PN 40), but with two different restorers, a natural (PN 38) and semi-RS restorer line – S1 (PN 50), showed similar heterosis effects, 108% for hybrid PN 54 and 110% for hybrid \( \text{F}_1 \) PN 55. Heterosis calculated as the mean value of seed yield of parents of \( \text{F}_1 \) hybrid with semi-RS restorer line was 124.7%, and for \( \text{F}_1 \) hybrid with natural oilseed rape restorer line, it was 112.2% (Table 4). The ability of seed yielding of two paternal lines was different: 30.7 dt\( \cdot \text{ha}^{-1} \) for S1 restorer line and 40.3 dt\( \cdot \text{ha}^{-1} \) for natural oilseed rape restorer line (Table 4).

![Fig. 3. Distribution of DH lines with the \( \text{Rfo} \) gene according to glucosinolate content.](image1)

![Fig. 4. Electrophoresis through a 1.8% agarose gel of RAPD-PCR products obtained with the primer OPC 02. R+: model plant with restorer gene; R-: model plant without restorer gene; S1, S2, S3, S4: double-low semi-RS DH plants tested for the \( \text{Rfo} \) gene marker (arrow shows polymorphic band associated with the \( \text{Rfo} \) alleles); M: molecular size marker (1 kb ladder).](image2)

![Fig. 5. Dendrogram of the genetic relatedness of RS lines (R34/13, R33/13), semi-RS DH lines (R12 125/13, S1, S2) and 96 parental lines of winter oilseed rape \( \text{F}_1 \) hybrids as shown by 344 AFLP markers.](image3)

### Table 3. Erucic acid and glucosinolate content in seeds of four selected DH lines with the \( \text{Rfo} \) gene obtained from semi-RS hybrids of winter oilseed rape

| \( \text{F}_1 \) hybrids | DH line with \( \text{Rfo} \) gene | Erucic acid content (% of all fatty acids) | Glucosinolates (\( \mu \text{mol g}^{-1} \) of seeds) |
|------------------------|----------------------------------|------------------------------------------|------------------------------------------|
| 1202/3 × RS 2 = 12r    | RS12/149=S3                      | 0.0                                      | 15.3                                     |
| 1215/4 × RS 2 = 17r    | RS17/48=S4, RS17/175=S1, RS17/322=S2 | 0.9, 0.0, 0.0                           | 4.4, 11.5, 11.6                         |
Discussion

The use of RS lines for the generation of hybrid varieties of *Brassica napus* L. is promising, because they represent a genetic resource that differs from that of the worldwide breeding material in this species (Girke et al. 2012b). Significant heterosis for seed yield in oilseed rape has created interest in the development of hybrid cultivars. The biggest constraint on the direct use of RS lines of oilseed rape in breeding programs is that their genotypes do not contain double-low quality traits. Additional approaches are therefore needed before the introduction of RS *B. napus* into breeding practice. One such strategy is the introduction of double-low quality restorer lines into RS oilseed rape, followed by in vitro androgenesis of F1 hybrids. Then, the desired DHs can be selected from the resultant population.

This strategy proved to be very effective in our hands. Using the linked PGI marker, it was possible to rapidly select lines with the Rfo alleles from a very large population of androgenic plants (Delourme and Eber 1992). The frequency of occurrence of lines with the restorer gene was similar to that obtained by routine crossing performed to obtain new lines with the Rfo gene (unpublished data). The presence of the restorer gene in four selected semi-RS lines (S1, S2, S3 and S4) was confirmed using the molecular marker OPC021150 (Delourme et al. 1994). One disadvantage of the semi-RS DH lines is that they form seeds poorly (Karim et al. 2014). Nevertheless, these lines produce high-yielding hybrids when crossed with adapted genotypes (Jesske et al. 2013). The major issue with the direct use of RS oilseed in breeding programs is their quality: the seeds have high glucosinolate content and high erucic acid content originating from *B. oleracea* and *B. rapa* (Girke et al. 2012b, Jesske et al. 2013, Rahman 2013) and, as expected, most of the semi-RS DH lines we obtained have this problem. In the DH populations studied, only about 10% contained zero erucic acid content in oil, while 10–20% had a glucosinolate content lower than 15 μmol g⁻¹ of seeds. In fact, only four lines were characterized by zero erucic acid content and glucosinolate content lower than 15 μmol g⁻¹ of seeds.

Information on genetic diversity is very important for any breeding program in *B. napus* and it can be obtained not only from data on agronomical traits, but also from molecular markers such as AFLPs. The dendrogram based on AFLP markers clearly indicated that both RS lines (R33/13, R34/13) and semi-RS lines (R12 125/13 and 00-quality lines S1, S2) formed a genetically distinct group from the 96 parental lines of winter oilseed rape F1 hybrids we tested. It is encouraging that, in general, crosses of lines located in different clusters resulted in greater yields yielded more than those from the same cluster (Riaz et al. 2001).

The heterosis effect of seed yield of F1 hybrids PN 54 and PN 55 measured in relation to the average values of the
parents was clearly greater for F₁ hybrid obtained from the semi-RS restorer line (PN 50) as the parental line. This is probably due to the larger genetic distance between the semi-RS restorer line and the CMS *ogura* line than the genetic distance between the natural restorer line and the same CMS *ogura* line. If the semi-RS restorer line yielded the natural restorer line level, a higher heterosis effect would be expected. The level of seed yielding of two F₁ hybrids with the same mother line was similar while the seed yields of the two paternal line were different.

Currently, the double-low quality semi-RS DH lines described here are being used for development of CMS *ogura* hybrids of winter oilseed rape. The results of field experiments with these new F₁ hybrids will be described elsewhere.

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