Doubled haploids as a material for biotechnological manipulation and as a modern tool for breeding oilseed rape (*Brassica napus* L.)

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Abstract

Haploids and doubled haploids (DHs) have been produced in *Brassica* ssp. using anther or isolated microspore cultures. Since 1982, when Lichter developed a method of isolated microspores culture, this technique has been constantly improved and modified. Haploids and DH of *Brassica napus* have been extensively used in genetic studies, such as gene mapping, location of quantitative trait locus, marker / trait association studies, and genomics. Furthermore, the oilseed rape haploid induction technique can nowadays be efficiently combined with several other plant biotechnological techniques, enabling several novel breeding achievements, such as mutation breeding, the breeding of hybrid varieties, genetic transformation, and resynthesis of *B. napus*. The development and improvement of *in vitro* androgenesis of oilseed rape allowed DH populations to be obtained on a large scale. Today DH lines are used in the genetic analysis of quantitative traits and to analyze the impact of the environment on the yield and quality of the seeds. Currently, DH technology has become more efficient and hence can be widely applied in winter oilseed rape breeding. Additionally, DH technology is used to shorten the process of breeding new varieties. An open pollinated *B. napus* cv. Monolit (Plant Breeding Strzelce Ltd., Co.) is the first Polish winter oilseed rape variety obtained using DH technology. The breeding cycle of this variety is about four years shorter compared to a classical breeding program. The second Polish cultivar obtained using DH technology, cv. Brendy (Plant Breeding Smolice Ltd., Co.) was developed in a similarly short period. In the breeding of new oilseed rape hybrid varieties, homozygous restorer lines are also exploited. Our basic research used oilseed rape DH technology with a focus on improving the qualitative traits of oilseed rape and is presented here, based on the major researches conducted worldwide as well as on the results obtained at the Plant Breeding and Acclimatization Institute – National Research Institute in Poznań.

Key words: *Brassica napus* L., oilseed rape, doubled haploids, markers-assisted selection, gene mapping, transformation, breeding

Abbreviations

AFLP	– amplified fragment length polymorphism	RFLP	– restriction fragment length polymorphism
CMS *ogura*	– cytoplasmic male sterility *ogura*	*Rfo* gene	– restorer gene of *ogura* cytoplasmic male sterility
DH	– doubled haploid	RIL	– recombinant inbred line
ISSR	– inter-SSR amplification	RS	– resynthesized *Brassica napus*
MDE	– microspore-derived embryo	SCAR	– sequence characterized amplified region
QTL	– quantitative trait locus	SNP	– single nucleotide polymorphism
PCR	– polymerase chain reaction	SRAP	– sequence-related amplified polymorphism
RAPD	– random amplified polymorphic DNA	SSR	– simple sequence repeat

Introduction

Oilseed rape (*Brassica napus* L.) is the third, after palm and soybean, most important source of vegetable oil in the world; it contributes significantly to the economy of many countries. Due to its economic value and high performance in an *in vitro* culture, oilseed rape is considered as one of the most suitable species that can be improved through biotechnology techniques. One of

the biotechnological methods most useful in basic researches and in plant breeding is androgenesis *in vitro* that aims at the development of haploids and doubled haploids (DH). Microspores isolated from the anthers of oilseed rape can be induced to develop into fully functional haploid embryos, instead of mature pollen grains. The ability of these cells to change their development process in response to environmental stimuli is an exceptional example of their totipotency.

The history of this method is almost a hundred years old. Since the first discovery of the spontaneous development of haploid plants in *Datura stramonium* in 1922 (Blakeslee et al., 1922), particularly after the discovery of *in vitro* androgenesis in anther cultures of *Datura* in 1964 (Guha and Maheshwari, 1964, 1966), the potential of haploids arose. Later, Zentkteler developed an *in vitro* production of *Atropa belladonna* L. haploid plants and in 1972 elaborated a method for obtaining embryos and plantlets from microspores in *in vitro* anther culture of *Lycium halimifolium* Mu, L. (Zentkteler, 1971, 1972).

Following those initial discoveries, many studies have been conducted to develop haploids in *Brassica* plants. Using anther or isolated microspore cultures, haploids and DH have been produced in *Brassica* ssp. *Brassica* anther culture was successfully used by Keller et al. (1975) and Thomas and Wenzel (1975). Alternatively, in 1982, Lichter developed an isolated microspore culture system for *B. napus*. These techniques have been gradually developed and constantly improved (Cegielska-Taras et al., 2002; Forster and Thomas, 2005; Palmer et al., 2005; Smýkalová et al., 2006; Ferri and Keller, 2007; rev. Pratap et al., 2009; rev. Touraev et al., 2009; Bohownik et al., 2011; Takahira et al., 2011; Murovec and Bohanec, 2012). Protocols for isolated microspore culture of *B. napus* vary among laboratories, especially for cultivating donor plants, harvesting buds, isolating microspores, inducing microspores for division, converting microspore-derived embryos to plantlets, and doubling of chromosome. Protocols for *B. napus* have been successfully extended to other *Brassica* species too.

Generally, the yield of androgenic plants in the microspore culture of *Brassica* species is higher than in the anther culture. In the method of microspore culture, every microspore is potentially capable of regenerating into a viable embryo, and each plant, therefore, represents the genetic variation that exists in the population of microspores. Plant regeneration occurs either by di-

rect embryogenesis or by secondary embryogenesis followed by organogenesis. A number of factors influence microspore embryogenesis including genotype, donor plant genotype growth conditions, the stage of explant development, pretreatment composition of the culture medium, and environmental conditions during the culture or the diploidization process. A large part of these factors have to be optimized for each microspore donor genotype (Cegielska-Taras, 2002; Babbar et al., 2004; Gu et al., 2004; Friedt and Zarhloul, 2005; Zhang et al., 2006; Gil-Humanes and Barro 2009; Pratap et al., 2009; Ferrie and Caswell, 2011; Ferrie and Möllers, 2011; Islam and Tuteja, 2012).

The key for increased regeneration efficiency during androgenesis largely depends on the control of two main developmental switches: the induction of microspore cell division and its ultimate commitment to the embryogenic pathway and diploidization rate (Maraschin et al., 2005).

Most plants obtained from the microspore culture of *B. napus* are haploid, which, in turn, must be diploidized. The frequency of spontaneous chromosome doubling is about 10-20% and varies among genotypes (Weber et al., 2005; Takahira et al., 2011); therefore, artificial chromosome duplication is required. The use of antimitotic substances immediately after the microspore isolation is the most effective method of artificial chromosome duplication (Zhou et al., 2002) and of obtaining microspore-derived embryos (Mohammadi et al., 2012). DH plants are produced by doubling the chromosome number of a haploid plant, while the conventional homozygous inbred lines are developed by selfing in successive generations. With the DH method, homozygous plants produced in one generation show a homozygosity of 100% compared to the conventional method, which results in an average level of homozygosity of 96.9%, that is, after five to six generations of selfing (Briggs and Knowles, 1967).

One of the principal advantages of haploid techniques is the fixation of segregating genotypes occurring at a lower frequency, in which the recessive gene coding for specific traits is combined in the homozygous condition (Friedt and Zarhloul, 2005).

Microspore culture plays a significant role in both fundamental and practical research. A number of review articles are found on the use of microspore culture and the resulting DH plants (Maluszynski et al., 2003; Forster and Thomas, 2005; Palmer et al., 2005; Friedt and

Snowdon, 2009; Pratap et al., 2009). This technique is simple and used in the production of *B. napus* DH plants; this method is highly preferred compared to classical breeding methods. Therefore, microspore culture is the method of choice in plant genetic research and breeding programs.

DHs of *B. napus*, besides being used for the production of homozygous lines, are also used for selecting lines that possess traits determined by recessive genes. DHs were also used for the basic research of physiology and biochemistry, induction and selection of mutants, improvement of plants through genetic transformation, and broadening the genetic diversity in the process of resynthesis of *B. napus* (Babbar et al., 2004; Palmer et al., 2005; Friedt and Snowdon, 2009; Pratap et al., 2009).

Basic researches on DH technology of oilseed rape and attempts to improve their qualitative and quantitative traits are presented based on the main researches conducted worldwide as well as on the results obtained at the Plant Breeding and Acclimatization Institute NRI in Poznań. The results presented here as well as the publications issued by our Institute were based on the data obtained from experiments performed using doubled haploid lines developed by a technology elaborated by Cegielska-Taras et al. (2002).

Development of molecular markers

Haploids can efficiently be used in basic studies, for example, to identify molecular markers associated with important traits. For the development of molecular markers, segregating populations for the trait of interest are required. Marker-assisted selection (MAS) allows the detection of desired traits in newly developed varieties at early stages of plant development in breeding programs.

Nowadays, new oilseed rape breeding programs that involve modern methods and techniques of biotechnology, plant physiology, molecular genetics and molecular biology have reached an advanced level. It is expected that new varieties will reveal an excellent result in terms of yield, quality characteristics, or disease and stress resistance. Different, sometimes antagonistic, directions of breeding are carried out in parallel and require increasing flexibility from breeders. To maintain competitiveness in this field, it is necessary to react rapidly to the demands of the oilseed market (Matuszczak, 2013).

However, the need for a rapid change stays in opposition to the traditional model of breeding, or the de-velopment of new varieties through the selection of certain genotypes spanning several growing seasons. To make the breeding process more efficient and effective and, as a consequence, to significantly accelerate the development of new varieties, molecular markers are used for the selection (Mohan et al., 1997; rev. Snowdon and Friedt, 2004; Mikołajczyk et al., 2008; rev. Matuszczak, 2013).

Because of their genetic homogeneity, DH lines are extensively used to determine single nucleotide polymorphisms (SNPs) that are specific for agronomically important traits and may be used as molecular markers in breeding programs. Several mapping populations were developed and association studies were performed along with positional cloning and genomic and transcriptomic sequencing experiments using *B. napus* DH lines to analyze the crucial genes and regulatory sequences for further application in basic and applied research (Snowdon and Friedt, 2004).

DH lines were developed to detect SNP in low-linolenic (LL) winter oilseed rape genotypes that were obtained by chemical mutagenesis at the Plant Breeding and Acclimatization Institute-NRI Poznan (Spasibionek, 2006). The LL DH line of winter oilseed rape, DH219/05, was selected from a population of DH lines developed by *in vitro* culture of isolated microspores from the LL M681 mutant line of *B. napus* ($C18:3 \leq 3\%$) obtained using ethyl methanesulfonate (EMS) (Spasibionek, 2006). Total DNA was isolated from a wild-type line and the LL mutant DH219/05 line and *FAD3* desaturase alleles from the homeologous A and C genomes of *B. napus* (*BnaA.FAD3* and *BnaC.FAD3*). Obtained DNA preparations were polymerase chain reaction (PCR) amplified, cloned, and sequenced (Mikołajczyk et al., 2010a). As a result, two statistically important point mutations were detected: one with a C to T transition in the mutant *bnaA.fad3* gene leading to a possible Arg to Cys substitution and another with a G to A transition in the 5′ donor splice site of the mutant *bnaC.fad3* gene, disrupting intron sixth splicing. In addition, the total RNA was isolated from the developing seeds of wild-type and mutant DH lines eight weeks after pollination. They were used for reverse transcription reaction to confirm the detected SNPs as well as to analyze the splicing variants for *bnaA.FAD3* and *bnaC.FAD3* (Mikołajczyk et al., 2010a). Functional genetic markers for monitoring *FAD3* alleles in breeding programs were developed, and the detection

of wild-type and mutant *FAD3* alleles comprised 1) independent PCR amplification of short fragments comprising SNPs and 2) the detection of SNPs based on the microsequencing method (SNaPshot) using allele-specific primers (Mikołajczyk et al., 2010a).

This unique SNaPshot assay enables a precise and unambiguous detection of the allelic variability in programs accompanied by MAS, including recombinant and hybrid breeding of the LL forms of winter oilseed rape.

DH populations to construct genetic maps

A DH population is ideal for genetic mapping (Maluszynski, 2003). Genetic mapping is one of the methods by which important crops are studied to identify the molecular markers which are linked to the valuable traits of the plant under study. In particular, when dealing with quantitative traits, influenced by many various genes, the localization of quantitative trait loci on the genetic map becomes a very important step in the genetic studies being performed.

For mapping *B. napus*, DH populations are commonly used, and it seems that their use has been consistently increasing (Lombard and Delourme, 2001; Parkin et al., 2005; Delourme et al., 2006; Kaur et al., 2009). A good illustration of this general tendency is the fact that among all *Brassica* mapping populations currently listed on the website Brassica.info, 70% (16) are DH populations and only 30% (7) are other types of populations (King and Baten, 2015). The most significant achievement of these studies is the construction of integrated maps of *B. napus*. A good example of such integration is the construction of a genetic map using three distinct DH populations and the subset of RFLP, SSR, RAPD, AFLP and SNP markers. The obtained BnaWAIT_01_2010a integrated linkage map contains 5,162 markers representing 2,196 unique loci (Wang et al., 2011). Other studies focused on the comparison of two maps based on DH (190 lines) and recombinant inbred line (94 lines) populations developed from the same cross (two diverse *B. napus* cultivars "Polo" and "Topas" were crossed). The maps were prepared based on SSR, SRAP, ISSR and SCAR markers. Data from the two genetic maps were used to construct a consensus integrated genetic map covering a total genome length of 2464.9 cM (Geng et al., 2012).

There are at least two reasons for the DH populations to be preferred during the process of map construction. Both reasons arise from the fact that in DH lines, all the loci exist in homozygous state. First, the DH population is practically immortal and it can be maintained over many years simply by selfing each line, without any genetic changes in subsequent generations. This makes it a good material for any genetic study or phenotypic analysis in various environments. Second, the DH population has a very simple pattern of segregation (the theoretical proportion of two alleles is 1:1 for each locus), which is of great value if the localization of QTLs for some complex traits is considered. When using dominant markers (e.g., RAPD or AFLP), the use of DH population is the only possible choice because dominant markers are unable to detect loci in the heterozygous state that are present in the F_2 population.

Isolated microspore culture method has been successfully used to produce DH plants and to the development of DH population (Matuszczak et al., 2001). A total of 320 DH plants thus obtained were derived from the F1 hybrid obtained from the crossing of parental lines DH-JN-86 (R1) × DH-ER$_2$-13/1 (R2). The seeds of the first parent had a low erucic acid and high glucosinolate content, whereas the seeds of the second parent had a high erucic acid and low glucosinolate content. The DH-JN-86 line was derived from the Jet Neuf variety, and the DH-ER$_2$-13/1 line was from our own breeding materials. The subset of 117 DH lines served as a basis for the construction of genetic map. This mapping population, as well as parental lines, were studied using RAPD (Matuszczak and Krzymański, 1999) and AFLP (Matuszczak, 2002) markers. Today, the obtained genetic map continues to consist of 75 markers grouped in 19 linkage groups. Since the parental forms exhibit variations in important agronomic traits such as erucic acid and glucosinolate contents in seeds, our research focused on the finding of QTLs for these traits. Fifty-eight various QTLs have been localized on the map so far (Matuszczak, 2010; Matuszczak et al., 2011).

The study on the yellow-seeded oilseed rape with improved seed meal quality is another example. This trait in *B. napus* is generally associated with a reduced seed coat thickness and consequent lowering of anti-nutritive crude fiber and phenolic compounds (Friedt and Snowdon, 2009). Yellow seeds do not occur naturally among the genotypes of *B. napus*. In various research groups, there are many forms of yellow oilseed rape lines obtained by transferring this trait from other yellow-

seeded *Brassica* species or obtained as mutations (Cegielska-Taras, 2002; rev. Rahman and McVetty, 2011). In genetic mapping, these yellow-seeded materials are used to identify the major genes that contribute to reduced seed coat and to develop markers related to this trait (Friedt and Snowdon, 2009). The Polish source of yellow-seeded *B. napus* was derived from the crosses of a spontaneous mutant with lighter seeds, found in the breeding material of a double low winter rapeseed, with segregating, spotted-seed-coat spring line, from earlier crosses of *Brassica napus* × *Brassica rapa var. oleifera*. The lines thus obtained were further improved with respect to quality traits and then those that showed a stable expression for yellow seediness were selected (Bartkowiak-Broda et al., 2011). Using androgenesis *in vitro* of isolated microspores of bright color lines, yellow-seeded DH lines were developed. Two yellow-seeded lines DH Z129 and DH Z114 were used for reciprocal crosses with black-seeded DH lines DH H5-105 and DH W40. Hence, for creating genetic maps of *B. napus*, two segregating populations consisting of 200 DH lines were used. All DH lines were analyzed using RAPD, AFLP, and SSR methods. As a result of the research, 889 polymorphic amplification products were obtained, but for the construction of a genetic map, only 349 of potential genetic markers could be used. Field experiments with these DH lines in two environments were carried out for four years to obtain the data of their phenotypic characteristics. These data will be used to create a genetic map of the yellow-seeded oilseed rape.

Mutagenesis and selection *in vitro*

A mutation technique was applied to improve the yield and quality of the crop and to develop resistance to diseases and pests. Many novel genotypes with desired traits have been obtained (Szarejko, 2003). In *B. napus*, microspores and haploid tissues have been used as a material for mutation (Kott et al. 1996; Cegielska-Taras and Szała, 2001; Szarejko, 2003; Li et al., 2005). A mutagenic treatment of a single, potentially totipotent haploid cell (e.g., an isolated microspore), followed by rapid differentiation in the embryo, represents a unique opportunity for screening homozygotic, non-chimerical M1 organisms for resistance to particular agents. Thus, the mutant production cycle can be shortened significantly. Haploid cells (microspores) or haploid tissue and selection can yield a plant population with genetically fixed

novel traits. The advantage of using a large-scale microspore selection system over a traditional somatic tissue selection system is evident (Cegielska-Taras and Szala, 2001). Mutation methods combined with *in vitro* selection, especially at the haploid level, to select genotypes with traits controlled by either recessive or dominant genes, improves the collection of desired mutants. In *B. napus*, haploid cells and tissues have been used to select mutants for herbicide resistance, disease resistance, lower glucosinolate content, fatty acid composition, long pods and short stems, and DH lines with improved resistance to *Sclerotinia sclerotiorum* and better agronomic characters (rev. Li et al., 2005).

The methods of early *in vitro* selection of microspore-derived embryo (MDE) genotypes discussed here appear to be promising for further optimization of DH technology in oilseed rape (Ferrie and Möllers, 2011). The number of PCR-based DNA markers for agronomically important traits is increasing and will allow for a more efficient MAS in segregating MDE populations at an early *in vitro* developmental stage (Ferrie and Möllers, 2011).

One such example is the application of DH techniques with a method of early selection for erucic acid in cotyledons of MDEs. In this study, the seeds of the selected DH lines had higher erucic acid, that is, more than 50%. The erucic acid contents (expressed in percent of the total fatty acid content) of the cotyledons of embryos and of the seeds derived from plant regenerated from the remaining parts of the embryos were highly correlated. Results showed that the selected DH lines had high erucic acid content along with high oil content (Cegielska-Taras et al., 1999).

Genetic modification of *B. napus*

Oilseed rape (*B. napus* L.) is particularly susceptible to *Agrobacterium tumefaciens*-mediated transformation allowing a considerable progress in the development of genetically modified varieties (Friedt and Snowdon, 2009).

The limitations of initial regeneration systems and problems with combining regeneration and transformation within the same cells are the major constraints hindering the advancement of plant transgenesis. The essential elements of successful plant transgenesis include the choice of cells and organs for recipients of the introduced gene and an efficient system for the selection of cells containing an integrated foreign gene in the ge-

nome. In order for the genes to be successfully transferred, the cells and tissues have to represent a high regenerative capacity to develop into a complete and normal transgenic organism. DHs obtained by tissue cultures of microspores and MDEs show uniformity, and as a complete homozygous, they can also be efficiently used in genetic transformation (Fukuoka et al., 1998; Nehlin et al., 2000; Takahata et al., 2005; Cegielska-Taras et al., 2008). The gene introduced into a haploid genome, which in this case was an MDE and subsequent chromosome doubling, gives rise to a homozygous transgenic oilseed rape (Cegielska-Taras et al., 2008b; Cegielska-Taras and Pniewski, 2011).

One of the first successful attempts to obtain a transgenic herbicide-resistant winter oilseed rape was the transformation of haploid MDEs using hypervirulent *A. tumefaciens*' EHA105 strain (Cegielska et al., 2008b). In this way, two biotechnological methods of oilseed rape – DH and genetic modification – were combined.

The elaborated MDE transformation method of winter oilseed rape allows to obtain genetically modified plants (Cegielska-Taras et al., 2008b; Cegielska-Taras and Pniewski, 2011). The main advantage of this method is that as a result of chromosome duplication in the transformed haploid, the introduced trait can be evaluated in a single step, as a transgenic homozygote. The production of transgenic homozygous oilseed rape provides a unique material for further studies of the inheritance and functionality of genes introduced through a subsequent generation, that is, for basic research, breeding programs, and utilitarian purposes. An alternative path to obtain oilseed rape with new quality traits is by transferring particular foreign genes to a plant genome.

Many projects have aimed at obtaining transgenic DH lines; one such example is an attempt to obtain DHs that are tolerant to drought stress. The genetic source that expresses resistance to drought in *B. napus* is unknown. Hence, there is a need to explore the mechanisms that increase the tolerance toward drought stress and also genetic manipulations and to widen the range of genetic variability of this species. It is expected that studies that focus on the above points will result in DH lines that are tolerant to water deficiencies.

Drought stress induces diverse changes at the cellular and molecular level and is associated with the production of plant phytohormones, especially abscisic acid (ABA). The key role in the regulation of ABA signaling is attributed to protein phosphatases such as ABI1 and ABI2 (ABA insensitive). Protein phosphatases, which are negative regulators of ABA signaling, interact with various targets to form a regulatory core modulating cellular processes and stress response pathways. Moreover, the concentration of calcium (Ca^{2+}) in the cytoplasm increases substantially in response to some factors of stress. The information about stress (an increase in the calcium concentration) is sensed by numerous proteins, including Ca-dependent protein kinases (CDPKs). In the study described here, the coding regions of the *Arabidopsis thaliana AtABI1* and *AtCDPK6* genes were inserted into the vector pPKGIB, and these constructs were introduced into *B. napus* via MDEs with *Agrobacterium* mediated transformation (Babula-Skowrońska et al., 2015; Olejnik et al., 2011). Three independent *AtABI1*-overexpressing *B. napus* DH lines such as 41, 42, and 45 were investigated with respect to their drought stress response. The authors found that the overexpression of *AtABI1* in *B. napus* negatively influences several important cellular processes such as relative water content (RWC), chlorophyll accumulation, and the expression profile of several ABA- and/or dehydration stress-inducible genes. These data show that the function of ABI1 protein phosphatase is highly conserved within the *Brassicaceae* family. Furthermore, it functions as a regulatory hub protein for dehydration stress responses in plants. Interestingly, there are six *ABI1*-related genes in the *B. napus* genome, which are formed by polyploidization during evolution. To investigate the functional diversification of the duplicated *BnaABI1* gene copies, two evolutionarily distant *BnaABI1* genes such as *BnaA01.ABI1.a* and *BnaC07.ABI1.b* were selected. The expression pattern of these genes showed differences indicating the differential involvement of the duplicated *BnaABI1* genes in the response of *B. napus* to drought. These results shed new light on the diverse role of the *ABI1* gene family in the drought response plasticity in the *Brassica* polyploid (Babula-Skowrońska et al., 2015).

Resynthesis of *B. napus*

The level of genetic diversity in double-low genotypes is relatively low. It is caused above all by intensive selection of genotypes in terms of two features associated: with improving oil, through eliminating erucic acid and with improving the meal, through the reduction of glucosinolates content. Today, oilseed rape breeders are

seeking genetic diversity in their breeding programs. A particularly successful method used to create a novel genetic variety is wide hybridization in a special resynthesis of *B. napus* from ancestral species *B. oleracea* and *B. rapa.* The resynthesized (RS) oilseed rape (*B. napus*) is potentially of great interest for hybrid varieties breeding because its heterosis effects are reported to be higher in crosses of genetically distant materials. A major problem using RS lines of *B. napus* in hybrid varieties breeding is the quality of their seed oil (high level of erucic acid) and seed meal (high glucosinolates content), which does not comply with the double-low quality of oilseed rape. Additional treatments are needed for breeding before introducing RS *B. napus* in practice. Szała et al. (2015a) described RS oilseed rape obtained through crosses between *B. rapa* ssp. *chinensis* var. *chinensis* (pak choy) and *B. oleracea* ssp. *acephala* var. *sabellica* (curly kale) using embryo rescue technique. Several RS oilseed rape lines obtained by this technique were crossed with double-low winter oilseed rape possessing *Rfo* gene for CMS *ogura* hybridization system. Large numbers of androgenic plant populations (semi-RS DH lines) were developed from F1 hybrids using a microspore *in vitro* culture method. The seeds of the obtained semi-RS DH lines were analyzed biochemically with regard to double-low quality (zero erucic acid and glucosinolates content). From the populations of the semi-RS DH, genotypes with double-low quality and *Rfo* gene were selected. Currently, the obtained semi-RS DH lines with *Rfo* gene and double-low quality are being used to produce new components of winter oilseed rape for the development of CMS *ogura* hybrids (Szała et al., 2015a).

Improvement of the quality of oilseed rape oil

At present, much attention is paid to the nutritional value of oilseed rape oil obtained from double-low cultivars.

With regard to nutrition, oilseed rape is a very valuable oil product, and it is not only a source of essential unsaturated fatty acids but also a source of natural antioxidants with other endogenous bioactive substances in seeds such as tocochromanols, sterols, beta-carotene, and phenolic compounds. The quality of *B. napus* oil is also enhanced by the presence of a series of beneficial hydrophobic compounds including carotenoids, tocochromanols, and phytosterols (Cegielska-Taras et al., 2008a, 2011). Carotenoids and tocochromanols are important

antioxidants and essential components of human diet providing a source of provitamin A (beta-carotene) and vitamin E (alpha-tocopherol).

A three-year study was performed on the range of the genetic variation among DHs to determine the effect of the genotype and the environment on tocopherol (T) and plastochromanol-8 (PC-8) content in winter oilseed rape (*B. napus* L.). The plant material consisted of 25 DH lines derived from the F1 hybrid obtained from the cross between two DH lines (Z × H), (Z-114 – yellow seeds; H_2-26 – black seeds) (Sieger et al., 2015). Among all the genotypes analyzed, 11 were selected as satisfactory and stable regarding tocopherol or PC-8 contents. From the breeding point of view, high heritability of traits guarantees the effectiveness of the selection and a rapid biological progress. The results of this study confirm the desirability of making changes to tocopherol and PC-8 contents using DH line technology. The lines obtained of the DH population derived from F1 plants of the cross between yellow and black seeds are characterized by different color of seeds, which correlated with their tocopherol homologues content. The high coefficients of heritability for α-T, γ-T, and PC-8 suggest a possibility of an effective selection based on the phenotype, allowing to obtain DH lines with a stable expression of traits. This suggests that α-T, γ-T, and PC-8 contents depend more on the genotype than on the environmental influence. This dependence is confirmed by a statistical analysis (Siger et al., 2015).

Phytosterols are produced by isoprenoid biosynthetic pathway and possess the properties of lowering LDL cholesterol. Thus, it would be highly desired to select high phytosterol content in oilseed rape oil (Amar et al., 2008). Recently, three QTLs for total phytosterol content were detected in a winter oilseed rape DH population, explaining 60% of the genetic variance for this trait.

The study on 44 DH lines of winter oilseed rape showed a large and significant variation of its total phytosterol and fat contents. The large genotypic differences in total phytosterol content indicate that an effective selection of genotypes with higher phytosterol content, in a cultivar development program, would be possible (Cegielska-Taras et al., 2011).

Hybrid breeding of winter oilseed rape

Currently, the breeding programs of rapeseed worldwide as well as in Poland are focused consistently on the

development of hybrid varieties which display an improved yield performance and better stability owing to the heterosis effect. The benefit of heterosis depends on the specific combining ability and genetic distance of parental lines which should be homozygous. Most breeding programs are based on cytoplasmic male sterility (CMS) systems, controlling cross pollination, while homozygous restorer lines are necessary to exploit heterosis effect. Today, DHs are widely used for producing homozygous parental restorer lines for breeding oilseed rape hybrids (Poplawska et al., 2007; Friedt and Snowdon, 2009).

In the CMS *ogura* system used in Poland as well as in many other breeding centers in the world, the restoration of fertility is achieved by a single restorer gene *Rfo*. The restorer has to be incorporated into a gene pool distant from the gene pool of male sterile lines. DH lines can be produced right away from F1 plants or starting from later selfing generations, which allows for some early testing (Frauen, 1994; Möllers and Iqbal, 2009; Paulmann and Frauen, 1997; Popławska et al., 2006).

DH lines possessing the *Rfo* restorer gene were selected after molecular analyses using specific biochemical and genetic markers. The biochemical assay used to detect the *Raphanus sativus* Pgi-2 isozyme locus closely linked to the *Rfo* gene (Delourme and Eber, 1992) was applied (Poplawska et al., 2007) in addition to other molecular markers. At the beginning, the OPC02 RAPD marker (Delourme et al., 1994) was used for monitoring the *Rfo* gene in parallel to the specific SCAR marker – for the CMS *ogura* (Sigareva and Earle, 1997). Then, the *Rfo* RAPD marker was converted to an SCAR one (Mikołajczyk et al., 2008), which was further combined with the "CMS" SCAR marker for developing a multiplex PCR assay (Mikołajczyk et al., 2010b), making the selection of DH lines with the *Rfo* restorer gene more time-, labor-, and cost-effective.

DH technology in winter oilseed rape breeding programs

Generally, because of the high response of *B. napus* genotypes to microspore culture techniques, the use of DH production has become a common practice in commercial breeding programs and has already resulted in numerous licensed varieties.

The most important benefits of using haploid plants in the breeding of new varieties are as follows: 1) homozygous recombinant line can be developed in one genera-

tion instead of numerous backcross generations; 2) selection for recessive traits in recombinant lines is more efficient since these are not masked by the effects of dominant alleles; and 3) varieties can be developed in reduced time period, for example, at least from 12 to 8 years.

Microspore culture is a useful tool in plant breeding because homozygous lines exhibiting the desired agronomic traits can be rapidly selected, which creates an opportunity for faster production of commercial cultivars. A breeding program of Canadian spring canola cultivar Quantum lasted five years (Stringam et al., 1995). It resulted in the selection of DH line which is characterized by a high yield and agronomically superior blackleg resistance. The other winter oilseed rape DH line, cultivar Mohican, was developed in the UK (Kučera et al., 2002). Currently, most of the Canadian spring oilseed rape cultivars that are grown are derived using DH technology (Ferrie and Möllers, 2011).

An open-pollinated cultivar Monolit (Plant Breeding Strzelce Ltd., Co.) is the first Polish winter oilseed rape variety obtained using DH technology (Cichy et al., 2005). The breeding cycle of this variety was about four years shorter than a classical breeding program. The second Polish winter oilseed rape variety being the DH line was registered as cv. Brendy (Plant Breeding Smolice Ltd., Co.). It is a DH line which was licensed in 2013. Both cultivars were obtained from isolated microspore culture *in vitro* at the Plant Breeding and Acclimatization Institute NRI in Poznań.

Numerous DH populations of winter oilseed rape obtained from an isolated microspore culture (Cegielska-Taras et al., 2002) have been widely used in various statistical researches, for example, to study the influence of environmental conditions on the stability of important yield components and the transgression effects of advantageous traits (Szała et al., 2015b).

Conclusions

Androgenesis *in vitro* is one of the most widely used culture techniques which has miscellaneous applications. The production of DH plants through a microspore culture is the milestone in the development of research on the genome of rapeseed at different levels and using different technologies. It is also used to determine the effectiveness of breeding programs.

It seems that the positive results of molecular studies on the identification of genes responsible for trigge-

ring the induction of divisions of isolated microspores of *B. napus* in *in vitro* culture may become another step in the improvement of DH technology.

Acknowledgment

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