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Chapter 13

Doubled haploids in breeding winter oilseed rape

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Abstract

The doubled haploid (DH) technique is routinely applied in winter rapeseed breeding programmes for the generation of completely homozygous lines. Although the method is quite old and perfected, there are still some problems that prevent a universal application of the technique in winter rapeseed breeding. Key problems remain the insufficient diploidisation rate, the low seed yield of primary haploids and the time-consuming and inadequate plantlet regeneration from the embryos. This contribution summarizes current applications and approaches to tackle above problems. Methods of early *in vitro* selection of microspore derived embryo genotypes are presented that appear promising for a further optimisation of the DH technology in rapeseed.

Keywords

Brassica napus, microspore derived embryos, *in vitro* selection, hybrid breeding, diploidisation, marker assisted selection, Canola

Introduction

It is generally accepted that the application of the doubled haploid (DH) technology to crop breeding has several advantages compared to conventional methods of producing homozygous inbred lines (Forster and Thomas 2005). Speeding-up of the breeding process is usually considered as the biggest benefit. Especially, increased selection efficiency can be achieved in early generations, which results from the greater proportion of additive genetic variation available for selection for quantitative traits due to homozygosity. Consequently, better discrimination between genotypes within crosses, better discrimination between crosses, and greater selection response across generations is possible (Snape 1997). Because of the reduced number of alternative genotypes for single and multiple alleles, smaller DH population sizes are needed compared to an F_2 population. Table 1 shows the minimum size of an F_2 and a DH population for the availability of a specific homozygous genotype in the case of unlinked loci (adapted from Jansen 1992). Clearly, DH technology requires considerably fewer genotypes compared to the F_2 when a large number of genes are involved in the inheritance of a trait. Nevertheless, a large number of DH lines need to be obtained from a cross segregating for a quantitative trait in order to obtain, with a high probability, the desired recombinant genotypes. Early simulation studies have shown that DH technology should be superior when selecting for quantitative traits with low heritability (Walsh 1974; Riggs and Snape 1977).

Table 1. Minimum population size of an F_2 and a DH for the availability of a specific homozygous genotype in the case of unlinked loci (α=0.95; adapted from Jansen 1992)

No. of loci	F_2 segregation	F_2 plants (n)	DH segregation	DH plants (n)
1	1:3	11	1:1	5
2	1:15	47	1:3	11
3	1:63	191	1:7	23
5	1:1023	3067	1:31	95
7	1:16383	49081	1:127	383

Theoretically, the use of DH lines should be less efficient in crosses where one of the parents is not adapted or lacks required traits, such as double low – canola – quality in rapeseed. In such materials, pedigree selection in early segregating generations is relatively easy, whereas an unselected DH population would contain a large proportion of unacceptable lines. Following a first yield testing, a large fraction of the material would be rejected, due to poor field performance. If, on the contrary, crosses among relatively narrow elite material are performed, visual selection in early generations of a pedigree programme is difficult and DH lines have the advantage that the genetic variance among them is higher than in any other generation.

There is still some debate on the question which generation should be used for the production of DH lines. In most cases F_1 derived DH lines are used to get a maximum gain in time, but DH production from F_2 or F_3 plants will allow for more recombination and for some pre-selection of the material (Snape 1997). The availability of only a single meiosis when applying the DH technology is a clear disadvantage, if undesirable linkages exist between traits under selection (Snape 1976). In this case, the pedigree or single seed descent (SSD) method has clear advantages. However, in many cases breeders have no prior knowledge of the degree of linkage between the genes under selection. Therefore, application of the DH method might be more suitable for crosses among adapted material and for species having a relatively large number of chromosomes, where a large part of the new variation would be released mainly as a result of re-assortment of chromosomes, and the importance of linkage would be relatively less than in species with fewer chromosomes (Riggs and Snape 1977). Rapeseed has with n=19 a comparatively large number of chromosomes. Zhao et al. (2006) detected fourteen QTL for oil content in a rapeseed DH population and found that they were distributed over 12 linkage groups, indicating that for this trait, assortment of whole chromosomes, and not crossing over, would be the primary source of variation in DH populations. However, usually several quantitative traits are selected for simultaneously.

Tissue culture constraints can be another hindrance and in some cases it may be difficult to produce and diploidize haploids in sufficient numbers to exploit adequately the hybrid. Hence, actual importance of the advantages and disadvantages of the DH technology depends very much on the crop species and on the efficiency, with which large numbers of DH-lines can be produced.

Applying DH technology in winter rapeseed breeding programmes

In rapeseed, homozygous lines are produced via the pedigree, the SSD and the DH method. With the SSD method, segregating generations are rapidly advanced in the glasshouse with no selection, each line being continued by a single seed in each generation. In spring rapeseed, the SSD method may be as quick as the DH method in producing nearly homozygous lines. However, in winter rapeseed, generation times are much longer due to its vernalisation requirement. Thus, application of the SSD method to winter rapeseed is not as fast as the DH method. The timetable of breeding winter rapeseed using conventional pedigree selection in comparison to DH is shown in Table 2. Using DH lines may shorten the breeding process by one or two years. However, the additional years required for pedigree selection can be used for a better evaluation of the year to year variation of the material in the field. Little is known regarding close linkage of traits relevant to cultivar breeding in rapeseed. Experimental comparisons showed that DH and SSD populations from the same crosses were very similar in mean and variance for agronomic and compositional traits (Charne and Beversdorf 1991).

Table 2 Timetable using DH lines in comparison to conventional line breeding in winter rapeseed (modified after Paulmann and Frauen 1991, Frauen 1994)

Year	Conventional	DH-standard	DH-fast
1	P_1 x P_2	P_1 x P_2	P_1 x P_2
2	F_1	DH line production	DH line production
3	F_2, observation plots	Multiplication	Observation plots
4	F_3, observation plots	Observation plots	Yield tests
5	F_4, yield test	Yield test	Yield tests
6	F_5, yield test	Yield test	Official yield trials
7	F_6, yield test	Official yield trials	
8	Official yield trials		

Haploid embryos of *B. napus* were first obtained from anther culture (Thomas and Wenzel 1975) and later also from isolated microspores (Lichter 1982). Since then, the method has been optimised. Presently, hundreds to thousands of microspore derived embryos (MDE) can be obtained from a single microspore preparation and the DH technology is widely applied in breeding programmes. A survey among eight companies having a winter rapeseed breeding programme in Germany showed that all of them use the DH method. On average the breeders applied the method to 33% of their crosses (Möllers, unpublished results). However, the extent to which the companies applied the technology to their crosses, varied between 10% and 100%. Seven companies did not apply the DH technology to their complete breeding programme. Being asked for their reasons, they indicated that the DH technology is too expensive (57%), that there is a lack of sufficient glasshouse space (43%), that the success of obtaining sufficient DH lines is not certain (29%) and that there are bottleneck problems with other work loads of the conventional breeding programme (29%). Furthermore, the eight companies specified the following major problems in the application of the DH technology: low seed yield from primary DH (88%), insufficient diploidisation (75%) and plantlet regeneration rate (63%), followed by synchronisation problems with the vegetation period (50%). Ideally, primary DH-plants are planted in autumn directly into the

field, to save labour and glasshouse space, to obtain a high seed yield per plant and to have a better first visual assessment of the plants performance. However, direct transfer to the field is only possible with plant material which is ready for transfer in August/September. Obviously, DH technology should become more efficient to enable an even wider application in winter rapeseed breeding. Key problems remain the insufficient diploidisation rate, the low seed yield of primary haploids and the time-consuming and inadequate plantlet regeneration from the embryos.

The problem of diploidisation and low seed yield of primary haploids

Rapeseed shows a low spontaneous diploidisation rate of microspore derived haploid plants in the range of 10 to 30%. Hence, primary DH plantlets need to be treated with colchicine to restore the fertile diploid genome. Diploidisation success is genotype dependent and rarely exceeds 50-70%. Furthermore, colchicine treatment of haploid plantlets in the glasshouse is laborious, requires substantial amounts of colchicine and causes a developmental retardation of the treated plantlets in the range of several weeks. Consequently, flowering and maturation of plantlets in the glasshouse is delayed and not synchronized, and glasshouse space is occupied for a longer period. Spontaneously diploid plantlets flower and mature first and give the full seed yield. Colchicine treated haploid plantlets flower late and give only a low seed yield due to their haploid/diploid chimeric nature. This often requires a seed increase generation in the glasshouse (see Table 1. DH-standard), before sufficient seeds are available for quality analysis and for sowing in observation plots in the field. The *in vitro* treatment of freshly isolated *B. napus* microspores with colchicine or other potent mitotic inhibitors helps to overcome several of the above mentioned problems. The treatment is performed with low dosages of colchicine or other mitotic inhibitors for a limited duration of up to three days. High diploidisation rates of up to 90% have been reported (Chen et al. 1994; Möllers et al. 1994; Hansen and Andersen 1996; Zhao et al. 1996). However, more recent results indicate considerable differences in the efficiency of diploidisation, which ranges between 30 and 95% (Zhou et al. 2002a; Weber et al. 2005; Möllers, unpublished results from survey among companies). This may partly be explained by genotypic differences in the sensitivity towards colchicine. But there is also a wide range of colchicine concentrations (10 to 1000 mg/l) and treatment durations (6 to 72 hours) applied. For practical applications, a further optimization of microspore protocols to obtain consistently high diploidisation rates with winter rapeseed breeding material is clearly desirable. This would render additional ploidy determination of regenerated embryos (Möllers et al. 1994) or plantlets unnecessary.

Diploidisation at the single cell microspore stage has additional advantages. *In vitro* colchicine treatment of microspores does not cause any developmental delay. On the contrary, colchicine treatment of microspores has been reported to improve embryogenic response by promoting symmetric division of the microspores (Zaki and Dickinson 1991, Iqbal et al. 1994). Regenerated plants are completely diploid, their development and maturation is synchronized and they give the normal seed yield one would expect from a diploid plant. This provides sufficient seeds for a direct testing of the material in observation plots in the field, making an additional seed increase generation unnecessary (see Table 2. DH-fast).

The problem of insufficient conversion of microspore derived embryos to plants

Only a small fraction of the MDE converts directly into plantlets. The larger fraction tends to form secondary somatic embryos on the hypocotyl and cotyledons, from which eventually shoots may regenerate. The low frequency of direct conversion of MDE to shoots requires

that a substantially larger number of MDE are sub-cultured to end up with the desired number of DH plants. Alternatively, several *in vitro* sub-cultures are required before normal plantlets are obtained, which can be transferred to the glasshouse. At present, very little is known about the endogenous factors that influence conversion of MDE to plantlets. Early colchicine treatment of isolated microspores in spring rapeseed, besides improving diploidisation and embryogenesis, also produced normal embryos developing directly into plantlets and avoiding cycles of secondary embryogenesis (Iqbal 1993; Zhou et al. 2002b). It has not been sufficiently investigated which of the different developmental stages of the MDE - torpedo, early, mid or late cotyledonary stage – is the best for transfer to solid medium to achieve direct plantlet regeneration. Results indicate that plantlet regeneration can be improved by adding phytohormones and vitamins to the regeneration medium (Tian et al. 2004), or by giving the MDE a cold or drought stress treatment (Zhang et al. 2006). However, usually only very few genotypes were included in those studies and the effect of a combination of several factors on plantlet regeneration has yet not been tested. Again, for routine applications in winter rapeseed breeding programmes further improvement of existing protocols are very much desirable.

In vitro selection

Assuming that the parents of a cross are different for a larger number of loci contributing to the expression of relevant traits, it is clear that breeders need to test many DH-lines to identify the desired recombinant line with a high probability (see Table 1). However, breeders usually aim to obtain only between 50 and 200 DH lines per cross (Möllers, unpublished results from survey among companies), which clearly appears to be too few. As mentioned above, thousands of microspore derived embryos can be obtained from a single microspore preparation. Usually only a smaller fraction of up to 300 of these embryos are sub-cultured *in vitro* to regenerate plantlets which are then transferred to the glasshouse for seed production. The subset of MDE genotypes used for plantlet regeneration represents a random sample of the total number of regenerated MDE, without any knowledge of their quality traits and agronomic performance. Thus many undesired genotypes go through plantlet regeneration and the costly glasshouse process. It also implies that valuable rare recombinant genotypes may be discarded at an early stage of *in vitro* culture. Any method that could be applied to determine useful agronomic or seed quality traits at an early stage of *in vitro* culture would definitely increase the frequency of valuable genotypes among the total number of regenerated plants. Selection *in vitro* for seed oil quality traits is possible in segregating populations of MDE (Albrecht et al. 1995). Fatty acid composition was determined by gas liquid chromatography (GLC) from single cotyledons, dissected from MDE. The rest of the MDE were maintained *in vitro* and regenerated to plantlets. Unequivocal selection for zero, intermediate and high erucic acid (Albrecht et al. 1995) and oleic acid contents (Möllers et al. 2000) was possible in segregating MDE populations. However, the application of this early *in vitro* selection system is limited to those traits which can be rapidly and cost effectively analysed and for which a close correlation between the MDE and the seeds from the regenerated plants has been shown. Alternatively, marker-assisted selection (MAS) could be applied at the MDE stage to screen a larger population for desired recombinants. One of the two cotyledons of the MDE can be dissected and used simultaneously for oil quality analysis and for DNA extraction (Horn and Rafalski 1992, Nath et al. 2007). Currently, only few trait specific molecular markers are available in rapeseed, but it is foreseeable that their number will increase substantially during the next years. A good example is the practised marker assisted selection for the Ogura CMS restorer

gene. The single restorer gene segregates one to one in a DH population, resulting in 50% of the lines being homozygous for the restorer gene. By applying MAS at the MDE stage, those 50% could be identified early and exclusively regenerated to plants.

DH technology in hybrid breeding programmes

A relatively new application for DH technology is the development of inbred lines for producing F_1 hybrid cultivars. Currently, mainly two male sterility systems are used for breeding hybrids in winter rapeseed. These are the cytoplasmic 'Ogura'-system and the genic 'MSL'-system. As mentioned above, restoration of fertility in the Ogura-system is achieved by a single restorer gene. In the MSL-system there is no specific restorer required, most of the genotypes being capable of restoration. Although it has been possible to obtain haploids from male sterile plants via microspore culture, they can not be used for breeding hybrid cultivars, because an isogenic maintainer has to be developed simultaneously by back-crossing. Consequently, DH technology can be meaningfully applied only to the male restorer gene pool. For the Ogura CMS-system, the restorer has to be incorporated into the gene pool. DH lines can be produced right away from F_1 plants or starting from later selfing generations, which allows for some early testing (Paulmann and Frauen 1991; Frauen 1994). Applying the DH technology will render repeated test crossing in later selfing generations unnecessary and simplifies maintenance breeding. Depending-on the stage at which the DH technology will be applied it will not save time, but the selection for combining ability will be more efficient due to the larger variance among DH lines.

Outlook

It is anticipated that further improvements of the microspore culture technique will allow in future a higher output of doubled haploid plantlets in practical breeding programmes. The number of PCR-based DNA markers for agronomically important traits are increasing and will allow for a more efficient marker assisted selection in segregating microspore derived embryo populations at an early developmental stage in the Petri dish.

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