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Development of synthetic *Brassica napus* lines for the analysis of "fixed heterosis" in allopolyploid plants

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Summary

Allopolyploids are widely spread in the plant kingdom. Their success might be explained by positive interactions between homoeologous genes on their different genomes, similar to the positive interactions between different alleles of one gene causing heterosis in heterozygous diploid genotypes. In allopolyploids, such interactions can also occur in homozygous genotypes, and may therefore be called "fixed heterosis". As to our knowledge, no experimental data are available to support this hypothesis. We propose an experimental approach to quantify "fixed heterosis" in resynthesised *Brassica napus* and the detection of loci contributing to "fixed heterosis" via comparative QTL mapping in *B. napus* and its parental species *B. rapa* and *B. oleracea*. In order to develop a genetically balanced material, interspecific crosses between 21 *Brassica rapa* and 16 *Brassica oleracea* doubled haploid or inbred lines were performed. In total 3485 vital embryos have been obtained from 9514 pollinated buds. The success of interspecific hybridisation was highly depending on the maternal genotype (*B. rapa*) and ranged from 0 to 1.18 embryos per pollinated bud. For the genetic characterisation of the *B. rapa* and *B. oleracea* lines, a dendrogram was constructed based on 273 RAPD markers. Thus a well-characterised material is now available, which is suitable to analyse the effects of "fixed heterosis" and the interactions between homoeologous genes in allopolyploid species.

Introduction

The aggregation of two or more genomes (polyploidy) has played a prominent role in plant evolution. Even plants with small genomes like *Arabidopsis thaliana* or rice, so far considered as classical diploids, seem to be ancient polyploids (Arabidopsis Genome Initiative, 2000; Goff et al., 2002). Therefore, some authors assume that nearly all higher plants have undergone at least one polyploidisation event in their evolution (Levy & Feldman, 2002). Polyploidisation may occur by multiplication of a single genome (autopolyploidy) or by the addition of different genomes by interspecific hybridisation (allopolyploidy). Many wild species as well as major field crops like wheat, oat, soybean, cotton and rape seed are the result of spontaneous interspecific hybridisation, showing the high potential of allopolyploid species. However, the causes of the success of allopolyploids are still not fully understood (Wendel, 2000). One explanation could be that favourable interactions may occur in allopolyploids between homoeologous genes located in the different genomes. These interactions are formally defined as epistasis but the biochemical and physiological mechanism of interactions between homoeologous loci of different genomes is probably very similar to interactions between two different alleles at the same locus. A favourable interaction between homoeologous loci should result in a permanently increased performance of allopolyploids compared to their parental species, even in homozygous genotypes. Therefore, such positive epistatic interactions can be called "fixed heterosis" because they

may be very similar to the interactions between alleles at the same locus causing "classical" heterosis and the fact that they are not lost by inbreeding.

"Fixed heterosis" as an evolutionary factor has been suggested early (e.g. Mac Key, 1976), but so far experimental data on the size of "fixed heterosis" are rare, since the diploid progenitors of allopolyploids are often unknown. In addition, experimental allopolyploids are often produced from heterozygous genotypes, hence these genotypes cannot be multiplied for a systematic comparison between allopolyploids and its progenitors. To overcome these shortcomings the allopolyploid *Brassica napus* (genome constitution AACC) is an ideal model to analyse "fixed heterosis", because its progenitors *Brassica rapa* (AA) and *Brassica oleracea* (CC) are well known, many homozygous lines of these two diploid species are available from breeding programs and the artificial resynthesis of *B. napus* from these lines is relatively easy to perform. In this contribution we want: (i) to propose a new experimental approach for a comprehensive analysis of "fixed heterosis" in *B. napus*, (ii) to characterise a set of doubled haploid (DH) lines as suitable base material for the application of this approach by molecular markers, and (iii) to present results on the hybridisation efficiency when producing resynthesised *B. napus* by combining the genomes of *B. rapa* and *B. oleracea*.

A new approach to analyse "fixed heterosis"

In *B. napus*, it is relatively simple to produce synthetic genotypes by crossing the parental species *B. rapa* and *B. oleracea* followed by embryo rescue and subsequent artificial chromosome doubling. To measure "fixed heterosis", the performance of these resynthesised lines could be compared with those of the parental *B. rapa* and *B. oleracea* lines. However, such a direct comparison is of very limited value because of two reasons: (i) accessions of *B. rapa* and *B. oleracea* are populations of heterozygous plants, but the resynthesised *B. napus* lines are completely homozygous as a result of the chromosome doubling, and (ii) *B. rapa* and *B. oleracea* are diploids whereas *B. napus* is allopolyploid. Therefore, in a direct comparison of resynthesised lines with their parents the effect of "fixed heterosis" is confounded by the effects of heterozygosity and polyploidy. The only possibility to overcome these problems is (i) to use DH lines (or at least highly inbred lines) of the parental species and (ii) to compare the allopolyploid resynthesised lines with autopolyploids produced from the parents (Figure 1). For these reasons resynthesised *B. napus* (allopolyploids) and autopolyploids should be produced from the same DH or inbred lines of *B. rapa* and *B. oleracea*.

For the identification of loci which contribute to the "fixed heterosis" a comparative QTL mapping could be performed. The principle of this "fixed heterosis"-QTL mapping based on three DH populations derived from two *B. rapa*, two *B. oleracea* and their resulting two synthetic *B. napus* lines is illustrated in Figure 2: in case the effects of homoeologous loci in the A and the C genome were additive (no interaction, no "fixed heterosis") a QTL detected in the A genome should have the same effect in the resynthesised *B. napus* population. A different situation arises when a favourable allele in the C genome is dominant over the homoeologous QTL locus in the A genome ("fixed heterosis"). In this case the QTL of the A genome would not be detectable in the *B. napus* population. Analogous to this situation QTLs could be studied which are only present in the C genome. Such comparative QTL mapping is of course only possible when the diploid and the allopolyploid DH populations have the same genetic constitution which is again only possible when the same homozygous lines are used for the production of allopolyploids and for the development of the DH populations.

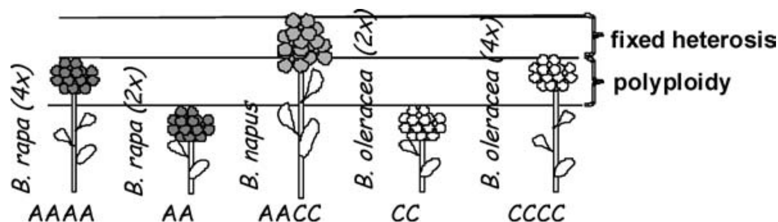

Figure 1. Material required to distinguish between the effects of autopolyploidy and "fixed heterosis" in allopolyploid *B. napus* (AACC); AA and CC denote homozygous genotypes of *B. rapa* and *B. oleracea*, respectively.

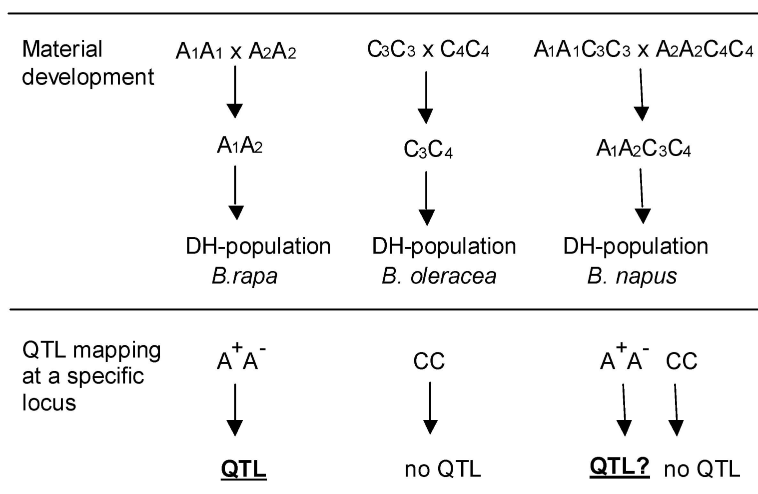

Figure 2. Principle of "fixed heterosis"-QTL mapping.

As an alternative approach the QTL mapping could only be performed in the diploid species, while in the allopolyploid species sets are constructed which represent either the negative or the positive allele (A^+A^+ vs. A^-A^-) at the QTL identified in one of the diploid species. In this approach a smaller number of genotypes could be tested more thoroughly in the field.

Characterisation of suitable base material

Material and methods

Initially, 21 *B. rapa* and 16 *B. oleracea* lines, hereafter called A1-A21 and C31-C49, were used as base material. The origin of these lines, their original denotation and additional information is listed in Tables 1 and 2. The collections of both species comprise genotypes of three subspecies that include also very closely related lines originating from the same cross.

For the genetic characterisation of the material, DNA was extracted from 14 *B. rapa* and 14 *B. oleracea* genotypes using the Dneasy® Plant Mini Kit from Qiagen (Hilden, Germany). Thirty-six 10-mer oligonuleotides from Operon Technologies (Almada, USA) were used for the subsequent PCR amplification. The reactions were carried out in a final volume of 25 μl containing 1 $\times$ Taq polymerase buffer without $MgCl_2$, 1 unit of Taq DNA polymerase (Fermentas, Lithuania), 0.2 mM of each dNTP (Fermentas, Lithuania), 0.4 μM of random primer, 3 mM $MgCl_2$ and about 50 ng of total genomic DNA. DNA amplification was performed in a PTC 100 thermocycler from MJResearch (Waltham, USA) programmed for an initial denaturing step of 0.5 min at 94 °C followed by 45 cycles of 1 min at 92 °C, 1 min at 35 °C, 2 min at 72 °C and a final step at 72 °C for 5 min. The PCR products were separated on one 1.5% agarose gel per primer and visualised under UV light after ethidium bromide staining.

Bands were scored as one for their presence or zero for their absence to generate a binary matrix. Genetic similarity values were computed based on Jaccard's coefficient of similarity (Jaccard, 1908). The data were subsequently used to construct a dendrogram using the unweighted pair group method using arithmetic averages (UPGMA) algorithm. All computations were carried out using the NTSYS-pc software ver. 2.1 (Rohlf, 2000).

Results

In total 273 RAPD loci were scored which corresponds to a mean of about 7.6 loci per primer. In *B. rapa* 197 RAPD loci produced a band with 169 being polymorphic. In *B. oleracea* only 136 loci produced bands with 89 being polymorphic. The cluster analysis resulted in a dendrogram shown in Figure 3. The different species as well as their subspecies formed discrete clusters. Variability was larger within *B. rapa* var. *rapa* and var. *pekinensis* than in subspecies *trilocularis* and larger within *B. oleracea* var. *alboglabra* than in subspecies *botrytis*. Genotypes originating from the same cross

Table 1. Description of the used *B. rapa* lines

Name	Original name	Source	Subspecies	Type
A1	DH1334	Canada – Plant Biotechn. Institute	var. *rapa*	DH
A2	DH1689	Canada – Plant Biotechn. Institute	var. *rapa*	DH
A3	DH1912	Canada – Plant Biotechn. Institute	var. *rapa*	DH
A4	RO18	UK – John Innes Center	var. *trilocularis*	Inbred
A5	6747-3016	Canada – Agri Food Canada	var. *rapa*	DH
A6	6748-21	Canada – Agri Food Canada	var. *rapa*	DH
A7	6748-1430	Canada – Agri Food Canada	var. *rapa*	DH
A8	6806-42	Canada – Agri Food Canada	var. *trilocularis*	DH
A9	7442-2942	Canada – Agri Food Canada	var. *rapa*	DH
A10	7485-15	Canada – Agri Food Canada	var. *rapa*	DH
A11	7545-11	Canada – Agri Food Canada	var. *rapa*	DH
A12	03-A6-901	China – Agric. Univ. Wuhan	var. *pekinensis*	DH
A13	03-A6-902	China – Agric. Univ. Wuhan	var. *pekinensis*	DH
A14	03-A6-904	China – Agric. Univ. Wuhan	var. *pekinensis*	DH
A15	03 TE 8	China – Agric. Univ. Wuhan	var. *trilocularis*	DH
A16	S2YS-Pb24-2/1	Germany – IPK gene bank	var. *trilocularis*	Inbred
A17	S2 Sampad	Denmark – Danisco Seed	var. *trilocularis*	Inbred
A18	S2 YS49-1	Denmark – Danisco Seed	var. *trilocularis*	Inbred
A19	S2 YS 50-1	Denmark – Danisco Seed	var. *trilocularis*	Inbred
A20	6806-43	Canada – Agri Food Canada	var. *trilocularis*	DH
A21	7442-1907	Canada – Agri Food Canada	var. *rapa*	DH

Table 2. Description of the used *B. oleracea* lines

Name	Original name	Source	Subspecies	Type
C31	SW505	Sweden – Svalöf Weibull	var. *botrytis*	DH
C32	SW506	Sweden – Svalöf Weibull	var. *botrytis*	DH
C33	SW507	Sweden – Svalöf Weibull	var. *botrytis*	DH
C34	SW508	Sweden – Svalöf Weibull	var. *botrytis*	DH
C35	SW510	Sweden – Svalöf Weibull	var. *botrytis*	DH
C36	SW513	Sweden – Svalöf Weibull	var. *botrytis*	DH
C37	SW514	Sweden – Svalöf Weibull	var. *botrytis*	DH
C38	SW517	Sweden – Svalöf Weibull	var. *botrytis*	DH
C39	SW518	Sweden – Svalöf Weibull	var. *botrytis*	DH
C40	SW521	Sweden – Svalöf Weibull	var. *botrytis*	DH
C41	SW522	Sweden – Svalöf Weibull	var. *botrytis*	DH
C42	A12	UK – John Innes Center	var. *alboglabra*	DH
C46	S2Bra165/83-3/1	Germany – IPK gene bank	var. *alboglabra*	Inbred
C47	S2C3-4-1	USA – Crucifer Genet. Cooper	var. *alboglabra*	Inbred
C48	S2Rah1-1	Denmark – Danisco Seeds	var. *alboglabra*	Inbred
C49	S2Rah2-1	Denmark – Danisco Seeds	var. *alboglabra*	Inbred

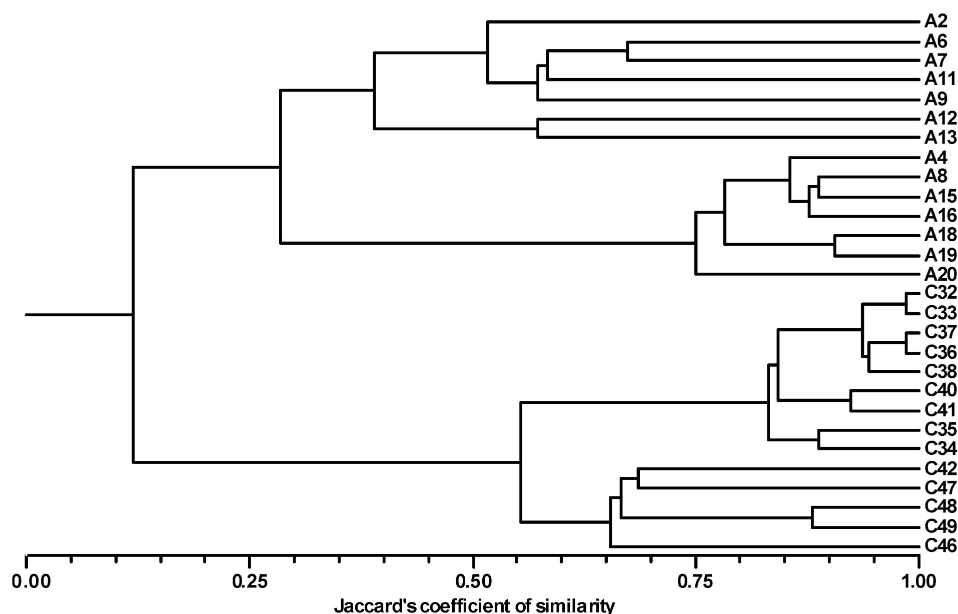

Figure 3. Dendrogram of 14 *B. rapa* (A) and 14 *B. oleracea* (C) genotypes based on 273 RAPD markers.

(e. g. A18/A19 and C32/C33) clustered together very closely. Consequently, there is a large variability of genetic distances available within the material.

Production of allopolyploids

Material and methods

Interspecific bud pollinations were made between all *B. rapa* and *B. oleracea* genotypes with *B. rapa* as female and *B. oleracea* as male in all combinations. In total 9514 buds were pollinated in 336 cross combinations (21 *B. rapa* × 16 *B. oleracea*) averaging 28.3 buds per combination. At least 275 and 500 *B. rapa* buds were pollinated per parental *B. rapa* and *B. oleracea* genotype, respectively. Twelve to 14 days after pollination, the developing siliques were removed, surface sterilised and the dissected ovules were aseptically cultivated on solid Murashige and Skoog (MS) medium at 24 °C with permanent light. The developing embryos and plantlets were transferred to new media every third week. Once roots emerged, up to five plantlets per cross combination were transferred into soil. After establishment, roots were washed and immersed in a 0.3% colchicine solution for 2 h for chromosome doubling. Finally, plants were transferred to soil again and grown in the greenhouse with 14 h/10 h light/dark periods. Fertile branches were bagged to prevent cross pollination and bud pollination was applied to self-incompatible genotypes.

The efficiency of hybridisation, hereafter called crossability, was calculated as the number of obtained vital embryos per pollinated bud. To test a potential influence of genotypes on crossability an analysis of variance was performed using the PLABSTAT software (Utz, 1992). For this purpose crossability values of different paternal genotypes (*B. oleracea*) were considered as replication for maternal genotypes *(B. rapa*) and vice versa. Thus the test on significance was very conservative since genotype by genotype interactions were regarded as trial error.

Results

From the developing siliques, 21060 ovules were dissected, cultivated *in vitro* and finally 3485 vital embryos were obtained. The interspecific crossability of genotypes varied from 0 to 1.18 embryos per pollinated bud with an average of 0.37 (Figure 4). Crossability was highly significantly affected by the parental genotypes. The variability of crossability between the genotypes was higher in the maternal parent (*B. rapa*) than in the paternal parent (*B. oleracea*) with coefficients of variation of 71 and 37%, respectively. Seeds from 197 different resynthesised *B. napus* genotypes have been

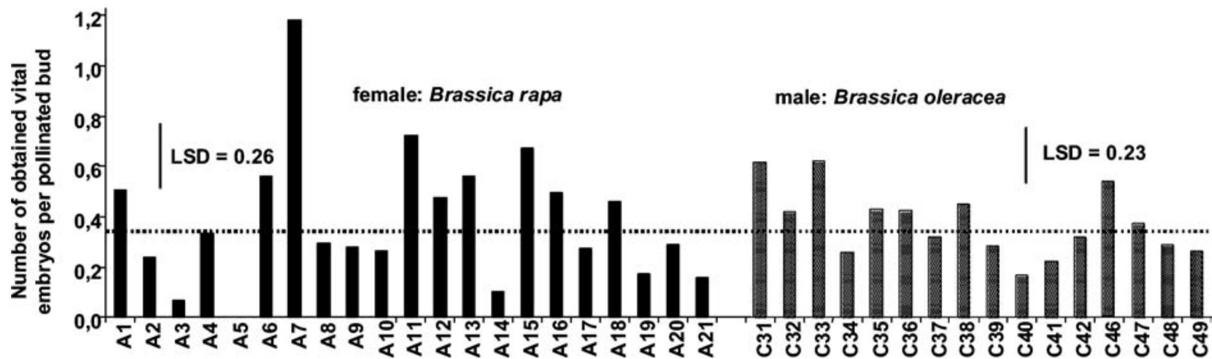

Figure 4. Crossability of different *B. rapa* and *B. oleracea* genotypes in interspecific crosses. Values for all genotypes are averaged over all crossing partners. Mean value for crossability was 0.37 (*dotted line*).

obtained so far. These include a core set of all 64 possible cross-combinations between 8 *B. rapa* and 8 *B. oleracea* lines.

Discussion

B. napus is a very suitable system to analyse "fixed heterosis" because both diploid ancestors, *B. rapa* and *B. oleracea*, are known and show a very large genetic diversity including wild and cultivated forms. Moreover it has been shown in a number of studies that the genomes of the two parental species show large homologies (e. g. Lagercrantz & Lydiate, 1996) and therefore a high number of homoeologous loci and interactions between those should be expected in the resulting allopolyploid. Another advantage of *B. napus* is that the resynthesis of *B. napus* by crossing *B. rapa* and *B. oleracea* has become relatively simple with the development of tissue culture techniques which have considerably increased the success of hybrid production since the first synthetic hybrids were produced by U (1935).

In our study *B. rapa* was used as female parent in all crosses because this cross combination usually yields in more interspecific hybrids per cross. In accordance to Lu et al. (2001) and Diederichsen and Sacristan (1994) we have found a highly significant influence of the maternal (*B. rapa*) genotype on crossability. However, genotypic differences were not related to the different subspecies. The observation of Matsuzawa (1983) that interspecific crossability was higher when self-compatible *B. rapa* genotypes were used as the female parent could not be confirmed here as genotypes A11, A12 and A13 are completely self-incompatible but showed a high crossability. It seems that inbreeding has also no generally negative effect on interspecific crossability because our results from crossing DH lines are comparable to values found in literature for crossing among heterozygous genotypes (Diederichsen & Sacristan, 1994; Lu et al., 2001).

So far, generally heterozygous genotypes were used for the interspecific hybridisation of *B. rapa* and *B. oleracea*. But only with *B. napus* genotypes resynthesised from homozygous lines a comprehensive and precise analysis of "fixed heterosis" is possible.

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