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The genetics of stamenoid petal production in oilseed rape (*Brassica napus*) and equivalent variation in *Arabidopsis thaliana*

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Abstract The agronomic potential of a *Brassica napus* variant with petalless flowers was compromised by an associated detrimental change in leaf morphology. Genetic analysis demonstrated the cosegregation of genes controlling both morphologies. Two *STAP* loci controlling the production of flowers with stamenoid petals were mapped to homoeologous locations in the genome of *B. napus*. The *STAP* loci were probably duplicate genes because they exhibited an epistatic interaction such that only plants homozygous for recessive *stap* alleles at both loci expressed the variant phenotype. The *CURLY LEAF* (*CLF*) gene of *Arabidopsis thaliana* pleiotropically influences both flower and leaf morphologies. The cloned *CLF* gene of *Arabidopsis* was homologous to a polymorphic *B. napus* locus coincident with one of the *B. napus STAP* loci. The possibility that *CLF* is a candidate gene for *STAP* suggests that the variant *stap* alleles of *B. napus* exert pleiotropic effects over both flower and leaf morphologies.

Key words Floral mutants · Pleiotropic effects · RFLP mapping · Candidate gene · Duplicate genes

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Introduction

The dense floral canopy produced by oilseed *Brassica* crops reduces the transmission of solar radiation to the photosynthetic tissues during the flowering period (Fray et al. 1996; Mendham et al. 1981). *Brassica* variants with reduced petal numbers have been identified (Buzza 1983), and physiological analysis has revealed the potential benefit of introgressing a petalless phenotype into oilseed rape (Fray et al. 1996; Rao et al. 1991). Here we describe a *B. napus* variant in which petals are converted into sterile stamens, the *stamenoid petal* (*stap*) variant. This morphology could improve radiation transmission as effectively as other petalless phenotypes, making its introduction into a superior agronomic background equally worthwhile. However, the *stap* variant possesses poor agronomic attributes, most notably deformed leaves and poor vigour. The aim of the present study was to use segregation analysis to investigate the genetic control of stamenoid petals and to use existing molecular-marker technology (Sharpe et al. 1995) to position the *STAP* loci on the genetic linkage map of *B. napus*. *B. napus* is genetically complex with an amphidiploid/allotetraploid genome and is thought to have originated from interspecific hybridisation between *B. oleracea* (C genome) and *B. rapa* (A genome) (U 1935). The ten linkage groups corresponding to the *Brassica* A genome and the nine linkage groups corresponding to the *Brassica* C genome have recently been identified in *B. napus* (Parkin et al. 1995).

Brassica napus and *Arabidopsis thaliana* are both members of the Brassicacae, and *A. thaliana* has become the subject of intense genetic investigation. The genomes of *Brassica* and *Arabidopsis* are collinear (Lagercrantz et al. 1996). A mutant phenotype similar to stap, namely curly leaf, has been identified in *A. thaliana* (Long et al. 1993; G. Coupland, unpublished). The *CURLY LEAF* (*CLF*) gene of *A. thaliana* has been

cloned recently (J. Goodrich, unpublished) making it possible to investigate whether *STAP* and *CLF* are homologous genes.

Materials and methods

Plant material

The *Brassica napus* line, N-o-11, was kindly donated by Cambridge Plant Breeders-Twyfords (Thriplow, Cambridgeshire, UK). Line N-o-11 exhibited two distinctive morphological characteristics: (1) flowers with sterile stamenoid structures replacing petals (stamenoid petals: stap) (Fig. 1A) and (2) curled leaves of reduced size (shrivelled leaves: shrl) (Fig. 1B). A doubled haploid line (N-o-11DH1) was produced from N-o-11 by microspore culture and pollinated with winter oilseed rape ('Tapidor') to establish whether the variant phenotype was dominant or recessive in an F_1 population. None of the 20 F_1 plants examined exhibited the stap or shrl morphologies, indicating that both characters were controlled by recessive alleles. A backcross generation (in which stap and shrl were segregating) was produced by pollinating a single F_1 individual with N-o-11DH1, and the Mapping Backcross Population of 207 plants derived from this cross was analysed. The same F_1 plant was used to pollinate 'Tapidor' and the Introgression Backcross Population of 60 B_1 plants derived from this cross was also analysed. Plants of the Mapping Backcross Population were grown in a lit glasshouse (16-h photoperiod) and flowered without vernalisation treatment. Plants exhibiting stap and shrl morphologies were identified visually.

RELP mapping

Restriction fragment length polymorphism (RFLP) marker technology and *Brassica* RFLP probes were as described by Sharpe et al. (1995). Southern hybridisation filters containing parental (N-o-11DH1 and 'Tapidor') DNA were used to screen for polymorphism and so identify informative probes. Three DNA fragments from *Arabidopsis thaliana* were also used as an RFLP probes; the *CURLY LEAF* (*CLF*) gene (Dr. J. Goodrich, unpublished), the *AGAMOUS* gene (Yanofsky et al. 1990) and the *APETALA*-3 gene (Jack et al. 1992). Segregation data was used to construct a linkage map using MAPMAKER V.1.9 (Lander et al. 1987). Linkage groups were assembled using a minimum LOD score of 3.0 and recombination frequencies were converted to map distances using the Kosambi (1944) mapping function.

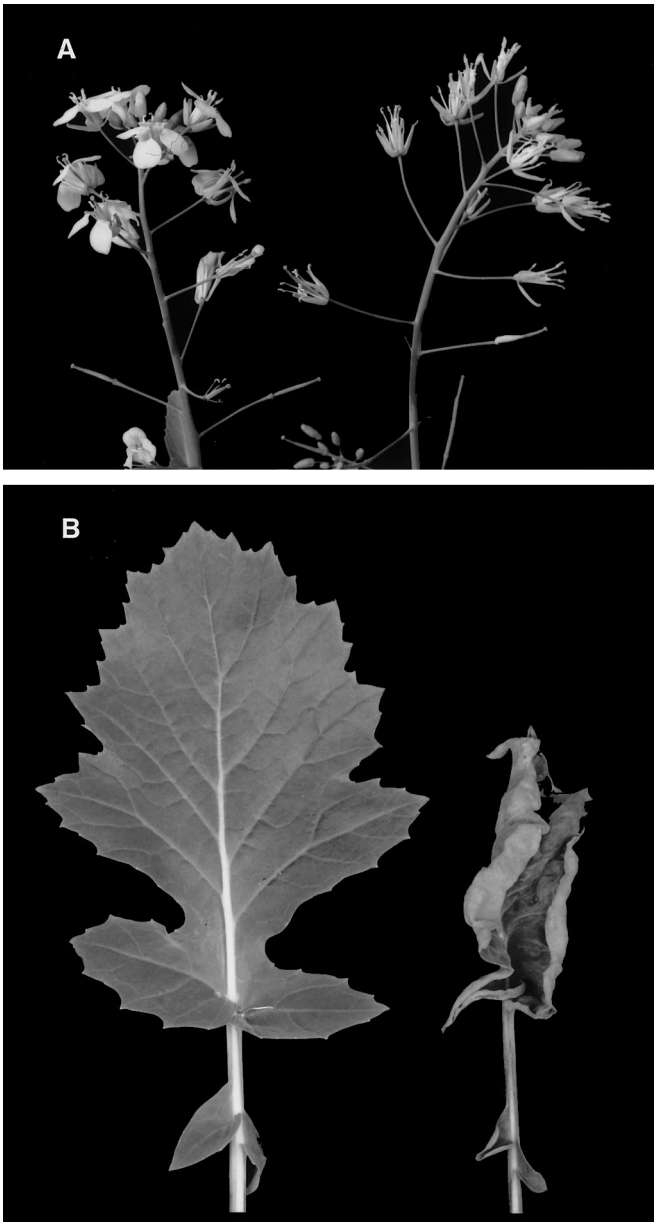

Fig. 1 Photographs of the oilseed rape variety 'Tapidor' and the morphological variant, N-o-11DH1. **A** Flower morphology of 'Tapidor' (*left*) and N-o-11DH1 (*right*). **B** Leaf morphology of 'Tapidor' (*left*) and N-o-11DH1 (*right*)

Results

Segregation of the stap and shrl phenotypes

Of the 207 plants from the Mapping Backcross Population 39 expressed both the stap (Fig. 1A) and shrl (Fig. 1B) morphologies. None of the backcross plants exhibited either stap or shrl phenotypes separately and there were no plants showing intermediate phenotypes. The segregation of the stap phenotype suggested that it was controlled by an epistatic interaction between two loci (Table 1), with segregation distortion at one or both loci resulting in a reduced frequency for the recessive alleles which promoted the stap phenotype.

RFLP analysis of the Mapping Backcross Population

The first sowing of the Mapping Backcross Population (83 individuals) was subjected to RFLP analysis using 54 informative *Brassica* probes. These probes revealed 76 polymorphic loci, and subsequent linkage analysis defined 10 linkage groups, five pairs of linked loci and seven unassociated markers (Fig. 2). Of the 76 loci 30 were equivalent to loci already positioned on more comprehensive maps of the *B. napus* genome (Sharpe et al. 1995; Parkin et al. 1995). These

Table 1 The probability of one-, two-, and three-gene models for the control of the stap morphology

Model[a]	Expected ratio[b]	χ^2	Probability (P)
Monogenic	1:1	80.7	$P < 0.001$
Digenic	1:3	4.19	$0.05 > P > 0.025$
Trigenic	1:7	7.16	$0.01 > P > 0.005$

[a] The models assume that homozygous alleles at one, two or three loci control the variant morphology and that no segregation distortion occurs at any of the loci controlling the trait
[b] Observed ratio 39:168 (or approximately 1:4)

common loci allowed the new map to be aligned with 14 of the 19 linkage groups defined by Parkin et al. (1995) (Fig. 2).

Mapping loci controlling the stap phenotype

Of the 83 individuals subjected to RFLP analysis, 19 exhibited the stap and shrl phenotypes and were thus expected to be homozygous for alleles from the N-o-11DH1 parent at the loci controlling the variant morphologies. Five of the RFLP-defined loci (*CLFe1*, *pN167e2*, *pO87e1*, *pW205e2* and *pN44e3*) exhibited homozygous N-o-11DH1 alleles in a high proportion of the 19 B_1 individuals expressing the variant morphologies. All five loci were located on linkage groups N4 and N14.

A further 30 individuals from the Mapping Backcross Population, including 17 which expressed the stap phenotype, were probed with markers on N4 and N14 to verify that these linkage groups contained the *STAP* loci. The proportion of the 36 B_1 plants with the stap phenotype that were homozygous for N-o-11DH1 alleles at each of the five previously identified loci is shown in Table 2. All five marker loci were significantly associated with one of two loci controlling the stap phenotype, one on N4 and the other on N14.

Further analysis of segregation data, for marker loci on N4 and N14 in all of the 113 B_1 individuals used for RFLP analysis confirmed that the stap phenotype could be accurately predicted from a model involving two epistatic *STAP* loci. The *STAP-1* locus mapped to the *pO87e1-pN167e2* interval of N14, 3.8cM from *pN167e2*, and the deduced *STAP-1* genotype invoked no double crossovers in the *pO87e1-pN167e2* interval (Fig. 3). The *STAP-2* locus mapped to the *pW205e2-pN44e3* interval of N4 and was coincident with *CLFe1* (a *B. napus* locus homologous to the *CLF* gene of *A. thaliana*: Fig. 3). The stap morphology was brought about by homozygous N-o-11DH1 alleles at both *STAP* loci (Fig. 3).

RFLP analysis of the Introgression Backcross Population

The Introgression Backcross Population was subjected to RFLP analysis as a prelude to the marker-assisted introgression of the *stap* alleles from N-o-11DH1 into 'Tapidor'. Sixty individuals were analysed with 41 RFLP probes, and segregation was scored at 60 polymorphic loci. The resulting genetic linkage data was readily aligned with the data from the Mapping Backcross Population because equivalent polymorphisms were scored in both populations (Fig. 2).

CLF as a candidate *A. thaliana* homologue of the *B. napus* gene controlling the stap phenotype

The *CLFe1* locus of *B. napus* was homologous to the *CLF* gene of *A. thaliana* and coincident with, and possibly equivalent to, the *STAP-2* gene of *B. napus* that controlled the expression of the stap and (possibly) the shrl morphologies. The *CLF* probe hybridised strongly with two *Eco*RI fragments in both the 'Tapidor' and N-o-11DH1 genomes (Fig. 4). One fragment from each parent segregated as RFLP alleles at the *CLFe1* locus, but the other fragment was the same size in both parents (monomorphic) and probably represented a second unmapped locus homologous to *CLF*. There were four additional weakly hybridising DNA fragments monomorphic in 'Tapidor' and N-o-11DH1 (Fig. 4).

In an attempt to map these additional loci, and particularly the locus assumed to be represented by the strongly hybridising monomorphic *Eco*RI fragment, we carried out RFLP analysis on DNA from 'Tapidor' and N-o-11DH1 (and several individuals of the Mapping Backcross Population) digested separately with *Eco*RV, *Bam*HI, *Hin*dIII, *Bgl*II and *Sau*3A and probed with *CLF*. In each case the majority of hybridising fragments were monomorphic, and no new polymorphic loci were identified. In a further attempt to map additional *B. napus* loci homologous to the *CLF* probe, RFLP analysis of two highly polymorphic populations of *B. napus* (Sharpe et al. 1995; Parkin et al. 1995) was carried out using the *CLF* probe, but again most of the hybridising fragments were monomorphic and no new loci were identified.

Discussion

The stap morphology was controlled by an epistatic interaction between two loci. Only individuals with homozygous recessive (N-o-11DH1) alleles at both *STAP* loci exhibit the stap phenotype. This type of genetic control is characteristic of a pair of duplicate genes catalysing the same biochemical reaction or

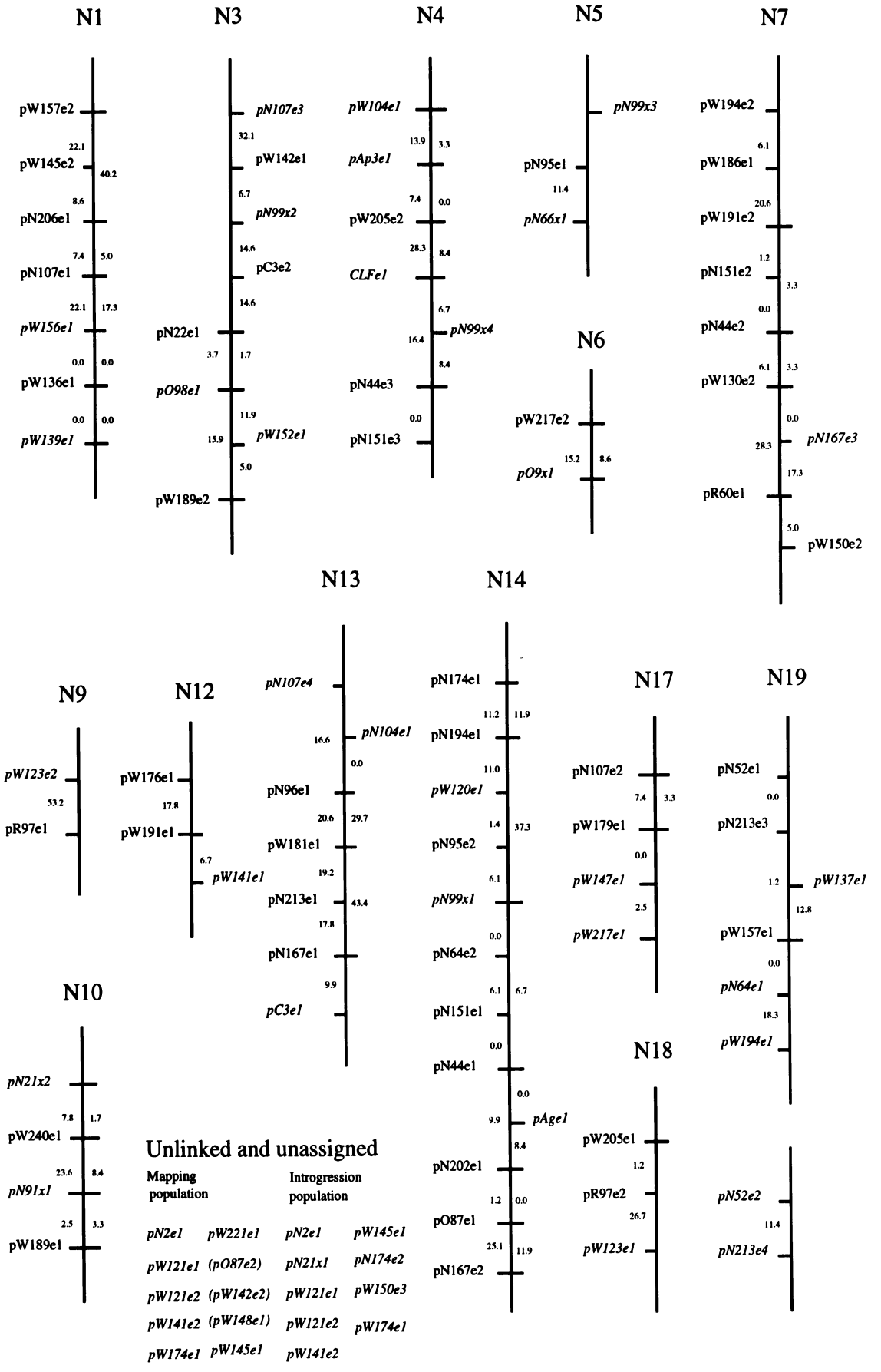
N1
pW157e2
22.1
pW145e2
40.2
8.6
pN206e1
7.4
5.0
pN107e1
22.1
17.3
pW156e1
0.0
0.0
pW136e1
0.0
0.0
pW139e1
N3
pN107e3
32.1
pW142e1
6.7
pN99x2
14.6
pC3e2
14.6
pN22e1
3.7
1.7
pO98e1
11.9
15.9
pW152e1
5.0
pW189e2
N4
pW104e1
13.9
3.3
pAp3e1
7.4
0.0
pW205e2
28.3
8.4
CLFe1
6.7
16.4
pN99x4
8.4
pN44e3
0.0
pN151e3
N5
pN99x3
pN95e1
11.4
pN66x1
N6
pW217e2
15.2
8.6
pO9x1
N7
pW194e2
6.1
pW186e1
20.6
pW191e2
1.2
pN151e2
3.3
0.0
pN44e2
6.1
3.3
pW130e2
0.0
28.3
pN167e3
17.3
pR60e1
5.0
pW150e2
N9
pW123e2
53.2
pR97e1
N12
pW176e1
17.8
pW191e1
6.7
pW141e1
N13
pN107e4
16.6
pN104e1
0.0
pN96e1
20.6
29.7
pW181e1
19.2
pN213e1
43.4
17.8
pN167e1
9.9
pC3e1
N14
pN174e1
11.2
11.9
pN194e1
11.0
pW120e1
1.4
37.3
pN95e2
6.1
pN99x1
0.0
pN64e2
6.1
6.7
pN151e1
0.0
pN44e1
0.0
9.9
pAge1
8.4
pN202e1
1.2
0.0
pO87e1
25.1
11.9
pN167e2
N17
pN107e2
7.4
3.3
pW179e1
0.0
pW147e1
2.5
pW217e1
N18
pW205e1
1.2
pR97e2
26.7
pW123e1
N19
pN52e1
0.0
pN213e3
1.2
pW137e1
12.8
pW157e1
0.0
pN64e1
18.3
pW194e1
pN52e2
11.4
pN213e4
N10
pN21x2
7.8
1.7
pW240e1
23.6
8.4
pN91x1
2.5
3.3
pW189e1
Unlinked and unassigned
Mapping population
pN2e1 pW221e1
pW121e1 (pO87e2)
pW121e2 (pW142e2)
pW141e2 (pW148e1)
pW174e1 pW145e1
Introgression population
pN2e1 pW145e1
pN21x1 pN174e2
pW121e1 pW150e3
pW121e2 pW174e1
pW141e2

Table 2 The proportion of the 36 stap plants from the Mapping Backcross Population that were homozygous for N-o-11DH1 alleles at loci on linkage groups N14 and N4

Linkage group	Locus	Number of stap plants[a]	χ^{2}[b]	Allele frequency[c]
N14	*pN167e2*	33	21.14***	0.42
N14	*pO87e1*	33	16.32***	0.46
N4	*pN44e3*	32	6.95*	0.56
N4	*CLFe1*	36	15.9***	0.52
N4	*pW205e2*	33	7.03*	0.58

[a] The mean number of stap plants with homozygous N-o-11DH1 allels was 19 for all other loci

[b] The probability of obtaining the various χ^2 values by chance is indicated by: $*P < 0.05$, $**P < 0.01$ and $***P < 0.001$

[c] The proportion of the 113 B_1 individuals from the Mapping Backcross Population that were homozygous for the N-o-11DH1 alleles

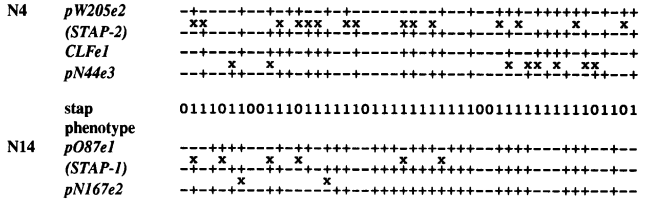

Fig. 3 The stap phenotype is brought about by homozygous N-o-11DH1 alleles at the *STAP-1* and *STAP-2* loci. Schematic representation of the deduced genotypes of the *STAP* loci and the actual genotypes of flanking RFLP-defined loci. *Rows* loci; *columns* B_1 individuals, — loci homozygous for the N-o-11DH1 allele, + heterozygous loci, *x* crossovers, *0* individuals with stamenoid petals, *1* individuals with normal flower morphology

controlling the same development step. *B. napus* is an amphidiploid/allotetraploid species with two constituent genomes, the C genome (probably derived from *B. oleracea*) and the A genome (probably derived from *B. rapa*), and the likely homoeologous relationships of *B. napus* linkage groups has been partially elucidated (Parkin et al. 1995). The alignment of the genetic linkage map derived from the *STAP* mapping population with more comprehensive maps of the *B. napus* genome (Sharpe et al. 1995; Parkin et al. 1995) demonstrated that *STAP-1* and *STAP-2* were located on homoeologous segments of linkage groups N14 and N4, respectively. N14 belongs to the *B. napus* C genome while N4 belongs to the *B. napus* A genome. The epistatic interaction between the *STAP* loci can be

Fig. 2 Combined genetic linkage map based on segregation in the Mapping (*left*) and Introgression (*right*) Backcross Populations. *Vertical lines* represent linkage groups numbered N1-N19 in agreement with pre-existing genetic maps of *B. napus* (Sharpe et al. 1995; Parkin et al. 1995). RFLP-defined loci are represented by the probe code followed by a *lower-case letter* (corresponding to the restriction enzyme that generated the polymorphism; *e Eco*RI, *x Xba*I) and a *number* (to distinguish different loci recognised by the same probe). Loci in *italics* have not been reported previously

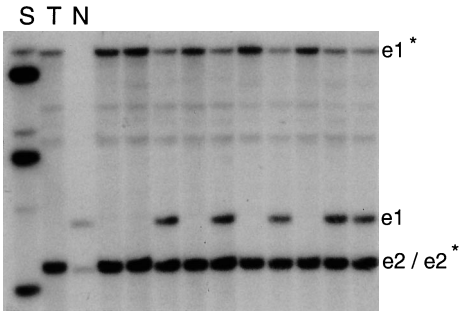

Fig. 4 Autoradiograph showing RFLP alleles segregating in 10 individuals from the Introgression Backcross Population and detected by the *CLF* probe. *Track T* ('Tapidor'), *track N* (N-o-11DH1) and the ten tracks representing individuals from the Introgression Backcross Population all contained total DNA digested with *Eco*RI. *Track S* contained a size standard with DNA fragments 27 kb, 18 kb, 10 kb, 7.6 kb, 6.3 kb and 4.4 kb in size. The *CLF* probe detected one polymorphic locus (*e1*) and a strongly hybridizing monomorphic band that probably represented a homoeologous locus (*e2*). Alleles from the 'Tapidor' parent are identified by an *asterisk*

explained by intergenomic complementation between a functional pair of duplicate homoeologous genes, one from each of the ancestral genomes.

Translocations involving homoeologous segments of related linkage groups have been identified in *B. napus* (Sharpe et al. 1995). Theoretically, the same rare allele can be acquired at a pair of homoeologous loci as a result of translocations mediated by homoeologous recombination. It was possible that the presence of the variant *stap* allele(s) at a pair of homoeologous loci was due to the translocation of a segment of one chromosome (carrying the variant *stap* allele) into the homoeologous position on the related chromosome containing the duplicate *STAP* locus. However, the loci flanking the *STAP-1* locus on N14 (*pO87e1-pN167e2*) and the loci flanking the *STAP-2* locus on N4 (*pW205e2-pN44e3*) mapped unambiguously to distinct linkage groups in a pair of reciprocal B_1 populations (the Mapping and Introgression Populations). This indicated that none of the four flanking RFLP markers possessed duplicate alleles (i.e. the same allele at a pair of homoeologous loci on N4 and N14) and demonstrated that none of the 4 flanking loci could have been involved in a recent homoeologous translocation. Furthermore, *CLFe1* and *STAP-2* were coincident (and possibly equivalent) and yet the N-o-11DH1 RFLP-allele at the *CLFe1* locus was present in a single copy (Fig. 4) and restricted to linkage group N4, again suggesting that the variant allele of the *STAP-2* locus on N4 was not duplicated on N14. These results suggest that the variant alleles at *STAP-1* and *STAP-2* arose independently and might indicate that mutant alleles are tolerated at a high frequency in *B. napus* because of intergenomic complementation.

None of the individuals in the Mapping Backcross Population segregating for stap and shrl exhibited

either variant morphology independently. This indicated that stap and shrl were either pleiotropic effects of the same variant alleles or under the control of tightly linked loci. The pleiotropic effects model would indicate that the *stap-1* and *stap-2* alleles of N-o-11DH1 are unlikely to be of agronomic benefit. In contrast, the genetic linkage model would suggest that a large recombinant population could contain individuals with stamenoid petals and normal leaves, i.e. individuals in which crossovers had broken the association between N-o-11DH1 alleles at resolvable *STAP* and *SHRL* loci.

The coincidence of the *STAP-2* and *CLFe1* loci of *B. napus* identified *CLF* as an *Arabidopsis* candidate homologue of the *B. napus* genes controlling the stap morphology. The transposon-induced *curly leaf* mutation of *Arabidopsis* exhibits both stap and shrl morphologies (Long et al. 1993; G. Coupland, unpublished), and both mutant phenotypes revert simultaneously upon excision of the mutagenic transposon (P. Puangsomlee, unpublished), indicated that stap and shrl are pleiotropic effects of a mutant *clf* allele in *Arabidopsis*. These considerations favour the pleiotropic effects model for the association of stap and shrl morphologies in *B. napus* and indicate that experiments to break the association between these two phenotypes are likely to be fruitless. This analysis of the genetic control of a petalless variant of *B. napus* and the identification of a corresponding candidate gene in *Arabidopsis* has demonstrated one way in which fundamental research into flower development in a model organism can assist in the design of experiments to improve the agronomic performance of related crop species.

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