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ORIGINAL PAPER

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The development of lettuce backcross inbred lines (BILs) for exploitation of the *Lactuca saligna* (wild lettuce) germplasm

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Abstract Backcross inbred lines (BILs) were developed in which chromosome segments of *Lactuca saligna* (wild lettuce) were introgressed into *L. sativa* (lettuce). These lines were developed by four to five backcrosses and one generation of selfing. The first three generations of backcrossing were random. Marker-assisted selection began in the BC_4 generation and continued until the final set of BILs was reached. A set of 28 lines was selected that together contained 96% of the *L. saligna* genome. Of these lines, 20 had a single homozygous introgression (BILs), four had two homozygous introgressions (doubleBILs) and four lines had a heterozygous single introgression (preBILs). Segregation ratios in backcross generations were compared to distorted segregation ratios in an F_2 population, and the results indicated that most of the distorted segregations can be explained by genetic effects on pollen- or egg-cell fitness. By means of BIL association mapping we were able to map 12 morphological traits and hundreds of additional amplified fragment length polymorphic (AFLP) markers. The total AFLP map now comprises 757 markers. This set of BILs is very useful for future genetic studies.

Introduction

When genetic studies are carried out to unravel the identity and function of genes behind specific plant traits, two types of traits can be discerned: the discrete trait, which is often explained by single genes following a Mendelian pattern of inheritance, and quantitative traits, which are explained by quantitative trait loci (QTLs) with complex patterns of inheritance. For an optimal genetic analysis of quantitative traits to be carried out, large segregating populations with good fertility, vigour, similar plant architecture and physiology and limited distorted segregation are required (Lander and Botstein 1989). Hundreds of genes for quantitative traits in crops have been mapped using QTL mapping procedures (Young 1996; Grandillo et al 1999; Van Berloo and Lindhout 2001). If segregating populations do not meet the prerequisites that allow QTL mapping, advanced backcross lines such as backcross inbred lines (BILs) can be an useful alternative (Zamir 2001). BILs are lines obtained by several generations of backcrossing with one of the parents (=recurrent parent) starting from the F_1 generation and ending with at least one generation of selfing. These lines have a high percentage (mostly higher than 90%) of the recurrent parent genome and a low percentage (mostly less than 10%) of the wild-parent genome. Each BIL intentionally contains one introgression segment, and a complete set of BILs should cover the complete genome of the wild species. For the purpose of genetic analysis, a complete set of BILs has several advantages over other types of segregating populations. (1) There is a high genetic and morphological similarity between lines that enables more precise estimates of quantitative traits. (2) BILs are homozygous lines, thereby enabling infinite replications of measurements and experiments, also in different seasons and environments. In this way, specific QTL × environment interactions can be studied accurately. (3) The interaction between several QTLs can be studied by intercrossing the BILs harbouring the respective introgression segments. (4) A practical advantage of BILs for commercial breeding purposes is that due to the low percentage of wild-parent genome, the introduction of an interesting trait into a commercial cultivar will be relatively straightforward and rapid.

However, genetic studies using a set of BILs also have some disadvantages compared to those in which other types of segregating populations are used. The development of a complete set of BILs is labour- and time-intensive and expensive: time- and labour-intensive because of the (research) time needed to produce many generations and money-intensive due to the need for many

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DNA marker fingerprints. Furthermore, if the trait of interest is explained by genes with epistatic effects, it might not even be revealed in the set of BILs.

Several sets of BILs have been developed for tomato and their wild relatives. These include *Lycopersicon pennellii* (Eshed and Zamir 1994), *L. hirsutum* (Monforte and Tanksley 2000) and *Solanum lycopersicoides* (Chetelat and Meglic 2000). In rice, a set of BILs was constructed between an *Oryza japonica* and an *O. indica* variety (Lin et al. 1996, 1998). All of these sets of BILs were developed using marker-assisted selection (MAS), but the number of backcrosses and selfings varied. For example, BILs were derived from $BC_1S_6BC_3S_1$ (Eshed and Zamir 1994), BC_2S_3 (Monforte and Tanksley 2000), BC_2S_6 (Chetelat and Meglic 2000) and BC_1S_5 (Lin et al. 1998). Several studies on these sets of BILs have already shown their usefulness for mapping and characterising genes and/or QTLs (Eshed and Zamir 1995; Lin et al. 1988; Monforte and Tanksley 2001; Zamir 2001).

We set out to develop a set of lettuce BILs with introgressions from the wild lettuce species *Lactuca saligna*. *L. saligna* is an interesting resource for resistance to lettuce downy mildew (*Bremia lactucae*). A survey on the biodiversity for *Bremia* resistance among several *Lactuca* species (including mainly *L. sativa*, *L. serriola*, *L. virosa* and *L. saligna*) indicated that only *L. saligna* is completely resistant to all *Bremia* races and can be considered a non-host (Norwood 1981; Gustafsson 1989; Lebeda and Boukema 1991; Bonnier et al. 1992). Despite many efforts, breeders have not been able to introduce this resistance into cultivated lettuce. We have made an effort to map this *Bremia* resistance by screening an F_2 population from a *L. saligna* × *L. sativa* cross (Jeuken et al. 2001). This F_2 population was not optimal material for mapping QTLs that were involved in *Bremia* resistance due to: (1) a limited population size that resulted from reduced germination and vigour (23% of the F_2 seeds did not develop into adult F_2 plants); (2) extreme variation in plant architecture and development among F_2 plants; (3) severe distorted segregations for several chromosome regions, which caused under-representation of certain genotypes; (4) sterility (37% of the F_2 plants were sterile), thereby preventing unbiased F_3 testing. Although we were still able to identify some QTLs, in view of the above-mentioned limitations we expected that not all QTLs for resistance to *Bremia* from *L. saligna* had been mapped in this F_2 population. Therefore, we developed a set of BILs with chromosome segments of *L. saligna* introgressed into *L. sativa*.

A precise selection tool is crucial for developing a set of BILs. For a rapid selection process and to assess the degree of coverage of the wild parent genome, a saturated and reliable genetic map consisting of equally dispersed high-throughput DNA markers is required. However, at the start of our backcross programme neither such markers nor a genetic map was available, and we chose to develop the first three backcross generations randomly without marker-assisted selection (MAS). Meanwhile, an interspecific amplified fragment length polymorphism (AFLP) linkage map was developed based on an F_2 population (Jeuken et al. 2001). This genetic map was immediately used for MAS in the fourth backcross generation (BC_4).

In the study reported here we developed a set of BILs with introgressions of *L. saligna* in a *L. sativa* background. Our goal was to obtain a set of BILs that covers the complete genome of *L. saligna* while each BIL contains only one homozygous introgression of *L. saligna*.

Materials and methods

Plant materials and development of BILs

The approach we used to develop BILs is depicted in Fig. 1. The same cross—*Lactuca saligna* CGN 5271 × *L. sativa* cv. Olof—was used for this backcross programme as was used for developing an F_2 population used in the construction of a genetic linkage map (Jeuken et al. 2001). A single F_1 plant was backcrossed with *L. sativa* as the paternal parent. The BC_1 generation was crossed reciprocally with *L. sativa* Olof to obtain the BC_2. BC_2 and BC_3plants were backcrossed with *L. sativa* Olof as the maternal parent to obtain the BC_3 and BC_4 plants, respectively. From the BC_4 generation we analysed 82 individuals with 267 AFLP markers. By using the genetic map of *L. saligna* × *L. sativa* (Jeuken et al. 2001), we obtained graphical genotypes from each BC_4 plant using the software programme GRAPHICAL GENOTYPING (GGT, Van Berloo 1999, http://www.dpw.wau.nl/pv/). Based on these graphical genotypes, the size, position and number of introgression(s) per plant were determined. Plants with one introgression of *L. saligna* were selected for selfing, and plants with two or more introgressions were selected for further backcrossing. For those regions of *L. saligna* that were lacking in this BC_4 generation, additional AFLP analyses were made on several plants of the BC_3, the BC_2 and all plants of the BC_1 generation to identify genotypes that contained one or more of the missing chromosome fragments. This extra backcrossing and selection on these plants was performed as described above for the BC_4 lines.

Phenotypic selection occasionally preceded AFLP genotyping when an obviously deviating phenotype was associated with a particular introgression fragment. At the final stage of BIL development, progeny of the selected selfed plants with one introgression were genotyped with AFLP markers and examined for a homozygous introgression. A plant with a single homozygous introgression was designated a backcross inbred line (BIL).

AFLP analysis

Leaf material was collected from 4- to 8-week-old plants. Genomic DNA was extracted from frozen leaves according to the procedure of Van der Beek et al. (1992) with some minor modifications: the DNA was washed overnight in 76% ethanol and 10 m*M* NH_4Ac, dried and dissolved in 100 µl sterile TE buffer (10 m*M* Tris-HCl, pH 8.0, and 1 m*M* EDTA). AFLP analyses were performed either with the radioactive label P^{33} (Jeuken et al. 2001) or with a Li-Cor detection system (Li-Cor, Lincoln, Neb.) using fluorchrome-labelled primers. AFLP analysis with the Li-Cor system was based on the AFLP reactions by a two-step amplification described by Vos et al. (1995) with some modifications: genomic DNA (250 ng) was digested with restriction enzymes and simultaneously ligated with adapters. PCR preamplification was performed in 20-µl volumes and consisted of 24 cycles of a 30-s denaturation at 94°C, a 30-s annealing at 56°C and a 60-s extension at 72°C. For the second PCR amplification we used *Eco*RI primers labelled with infrared dye IRDye 700 or IRDye 800 (Li-Cor). A 5-µl aliquot of the diluted secondary template was mixed with 50 ng unlabelled *Mse*I-primer, 0.5 pmol IRD700-labelled *Eco*RI-primer or 0.6 pmol IRD800-labelled *Eco*RI-

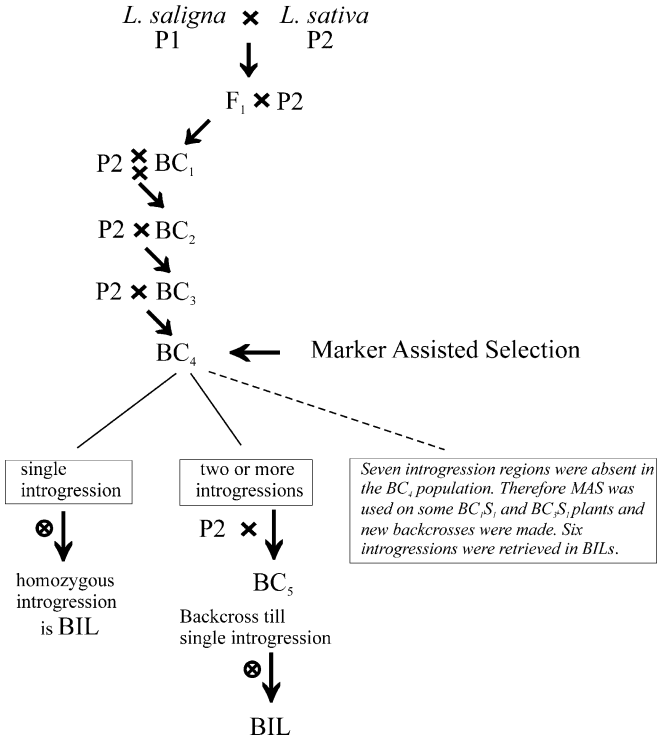

Fig. 1 Backcross and selection program for the development of a set of BILs. The *double cross symbols* indicate reciprocal crosses

primer in 0.2 m*M* of all four dNTPs and 0.2 U *Taq* polymerase (SuperTaq from Enzym Technologies) in PCR buffer (Superbuffer). AFLP reactions were performed following the touchdown PCR profile. For gel electrophoresis, 0.5–0.7 µl from each sample was loaded onto a 5.5% denaturing polyacrylamide gel (5.5% Ready-to-Use Gel Matrix, KB Plus, Westburg).

The majority of the markers in the AFLP analyses were scored dominantly, except for the analyses of the BC_4generation, where all markers were scored codominantly by Keygene (The Netherlands), using QUANTAR software (developed at Keygene).

Genotype presentation

During MAS all plants were genotyped with an average of 220 markers using six to eight AFLP primer combinations. When a plant with a single introgression was obtained, an extensive AFLP marker analysis was performed with a minimum of three extra primer combinations to once again verify the absence of other introgressions. The final set of lines was analysed yet again with all 12 AFLP primer combinations. For the graphical genotype analysis we used the genetic linkage map of *L. saligna* × *L. sativa* with 476 AFLP markers, an average spacing between markers of 1.8 cM and a maximal distance of 16 cM (Jeuken et al. 2001).

Results

Backcross programme

The BC_1 generation was obtained from a single F_1 plant by backcrossing with *L. sativa* Olof. Backcrosses on 11 random BC_1 plants yielded the BC_2 generation. Backcrosses on 13 BC_2 plants, descending from all 11 BC_1 parents, yielded the BC_3 generation. Seventeen random BC_3 plants, descending from all 13 BC_2 parents were backcrossed to obtain the BC_4 generation. All of the backcrosses, from the F_1 to the BC_4 generation, were made randomly—without selection for genotype or phenotype—because at the time the backcrosses were carried out a linkage map of *L. saligna* × *L. sativa* was not available. At the time that the BC_4 generation was obtained a linkage map had been constructed and, consequently, genotype analysis and selection became possible.

Eighty-two individuals from the BC_4 generation were analysed with 267 codominant AFLP markers. The percentage of the recurrent parent genome present in the BC_4 plants ranged from 77% to 100% (Fig. 2). The average proportion of recurrent parent genome per BC_4 plant was 95%, which did not deviate much from the theoretically expected 97% (31/32).

Based on the graphical genotypes, 63–69% of the *L. saligna* genome was present in the BC_4 (Fig. 3). The 6% inaccuracy was due to the distances between two markers flanking a recombination event (between the outermost analysed marker in the introgression and the first adjacent analysed marker outside the introgression). Between 30% and 36% of the *L. saligna* genome was missing and had to be retrieved from earlier generations (Fig. 3). Therefore, AFLP analyses were performed on 49 BC_3 plants. The only introgression that was lacking in the BC_4 and which could be retrieved from a BC_3S_1 plant was a fragment of chromosome 8 from 45 cM to 84 cM. The other introgressions that were lacking in the BC_4 were also absent in the BC_3 generation (data not shown).

To recover backcross lines with the missing introgressions we had to genotype all 12 individuals of the BC_1 generation using 125 dominant AFLP markers. The average proportion of recurrent parent genome per BC_1 plant was 74±9%, which did not deviate much from the theoretically expected 75% (Fig. 2). Graphical genotype analyses on the BC_1 plants showed that the complete genome of *L. saligna* was covered in the 11 BC_1 plants that had been backcrossed to BC_2 (Fig. 3). Chromosome

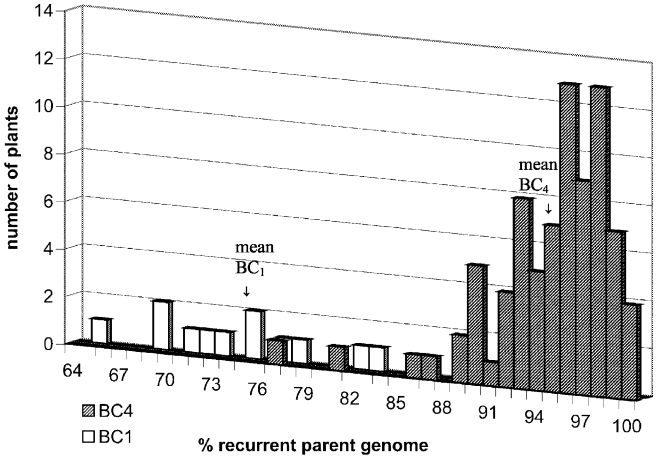

Fig. 2 Distribution of the percentage of recurrent parent genome values in the BC_1 and BC_4 generations. *Lactuca sativa* Olof is the recurrent parent and *L. saligna* CGN 5271 is the wild parent

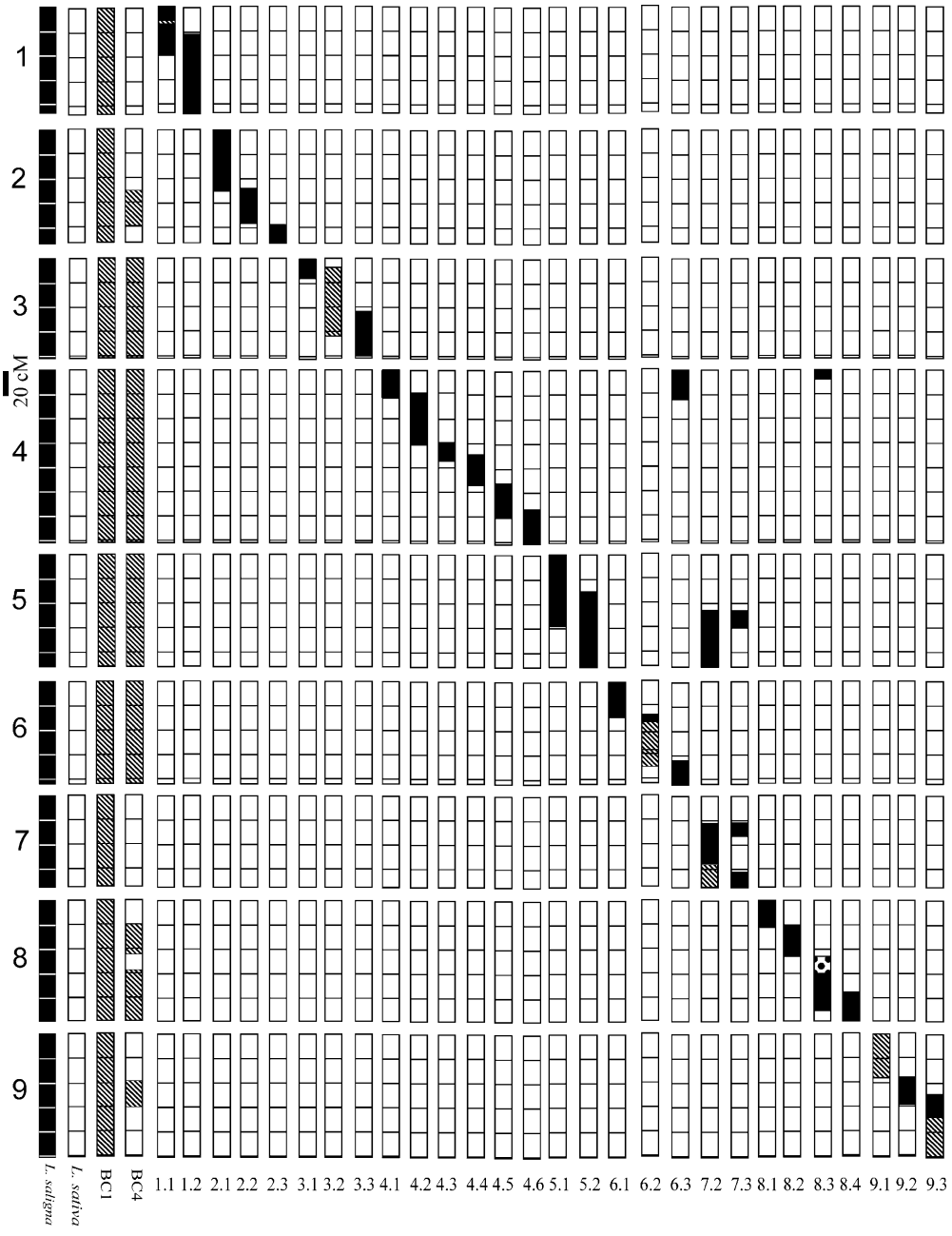

Fig. 3 Genome coverage of the BC_1 and the BC_4 generations and the genotypes of 28 backcross lines (*lines 1.1–9.3*) that cover more than 96% of the *L. saligna* genome. *Vertical bars* represent the nine chromosomes of lettuce. The chromosomes are segmented in 20-cM intervals that are delimited by *horizontal lines*. The genomes of the BC_1 and BC_4 populations and of the 28 backcross lines are indicated in *black*, *white* and *diagonal stripes*: *white* homozygous *L. sativa* Olof, *black* homozygous *L. saligna*, *diagonal stripes* heterozygous. *Dot* indicates an unknown genotype as not enough markers were analysed in that region. Introgressions of *L. saligna* are shown until the outermost analysed marker. Therefore, this is a minimal representation of the genome coverage as the genotype between the outermost marker of the introgression and the first adjacent marker outside of the introgression was not identified and still may contain *L. saligna* chromosome fragments

regions of *L. saligna* were presented in at least one BC_1 plant and in at most ten BC_1 plants. The introgression regions that were the most underrepresented in the BC_1 plants were: chromosome 2 (0–23 cM) and chromosome 7 (0–25 cM), present in one plant only; chromosome 9, present in two progeny plants only. Six introgressions that were lacking in the BC_4 generation could be retrieved from BC_1S_1 plants. The region on chromosome 7 from 0 cM to 25 cM was not retrieved. This introgression was present in one BC_1 plant but was absent in all eight genotyped BC_1S_1 plants. So, as the BC_1plants were lost, this chromosome fragment could not be retrieved anymore.

The selected BC_3S_1 and BC_1S_1 plants were backcrossed and selected for using MAS to develop BILs containing these introgressions.

A set of lines with a minimal number of introgressions and maximal genome coverage

A selection was made for the minimal number of lines with both a minimal number of introgressions and a maximal coverage of the homozygous *L. saligna* genome. This resulted in 28 lines that covered at least 96% of the *L. saligna* genome (Fig. 3). Twenty-four lines were homozygous, and the introgressions covered a total of 77% of the *L. saligna* genome. These 24 lines consisted of 20

BILs (1.1, 1.2, 2.1, 2.2, 2.3, 3.1, 3.3, 4.1, 4.2, 4.3, 4.4, 4.5, 4.6, 5.1, 5.2, 6.1, 8.1, 8.2, 8.4 and 9.2) harbouring one single introgression and four doubleBILs (6.3, 7.2, 7.3 and 8.3) containing two homozygous *L. saligna* introgressions. On average, each BIL contained 4% (=33 cM) of the *L. saligna* genome. All introgressions in these BILs were homozygous. There was only one exception for a very small region of 2 cM (from 11.5 to 13.5 cM) in BIL 1.1 that was heterozygous and still segregating even after several rounds of selfing. Three lines (3.2, 6.2 and 9.1) had a single heterozygous introgression. The progeny of these lines has been extensively genotyped in search for a plant with a homozygous introgression, but this genotype was not found. Therefore, these lines are designated "pre-BILs". Line 9.3 had a single introgression that was partially homozygous and partially heterozygous. No BIL that covered completely the bottom part of chromosome 9 was found. PreBILs 3.2, 6.2, 9.1 and 9.3 cover 18% of the *L. saligna* genome.

Distorted segregation ratios

During development of the BILs, large numbers of progenies of several backcross lines enabled us to compare segregation ratios over several generations. A preference for *L. saligna* alleles or for *L. sativa* alleles was sometimes observed. This influenced the number of generations needed for BIL development. A particularly severe preference for *L. sativa* alleles at some loci complicated the development of a BIL with a homozygous introgression from *L. saligna* at these loci. This phenomenon was observed for the following genome regions: chromosome 1 (0–21 cM), chromosome 3 (7–45 cM), chromosome 6 (31–61 cM), chromosome 7 (45–72 cM), chromosome 9 (0–35 cM, 67–101 cM). For all of these regions more than 50 plants from a progeny after selfing were analysed, and we never found a plant with a homozygous introgression (BIL) in any of the 50. An extra effort was made to find a BIL for the regions on chromosomes 1 and 7 as they contain QTLs for resistance to lettuce downy mildew (*Bremia lactucae*, Jeuken and Lindhout 2002). We ultimately found a BIL for these two chromosome regions after analysing more than 200 plants by AFLP.

Some of the distorted segregations of introgressions observed in the backcross lines were similar to those observed in the F_2 population—for example, the preference for *L. sativa* alleles of a fragment (45–72 cM) of chromosome 7 and fragments (0–35 cM, 67–101 cM)) of chromosome 9. Other distorted segregation ratios in backcross lines were unexpected as they showed a normal segregation in the F_2 population (n=126). This occurred for chromosome 1 (0–21 cM), chromosome 3 (17–45 cM) and chromosome 6 (31–61 cM).

Some of the distorted segregation ratios in the backcross lines only appeared when the backcross line was used as the paternal parent in the backcrosses. For example, the region on chromosome 5 from 0 cM to 13 cM showed a severe preference for *L. saligna* alleles when used as a pollen donor and normal segregation when used as an egg-cell donor. The top region of chromosome 9 from 0 cM to 33 cM also showed a skewed segregation with a preference for *L. sativa* alleles when used as the paternal parent but showed a normal segregation when used as the maternal one.

Morphological traits

During the development of the BILs some morphological characteristics from *L. saligna* became obvious within segregating progenies and between backcross lines. Some of these traits were easily mapped since they were invariably associated with *L. saligna* alleles of particular AFLP markers. Accordingly, these were used as easy scorable morphological markers (Table 1). For example, in *L. sativa* Olof the leaf apex is round, as in most *L. sativa* cultivars, while in *L. saligna* the leaf apex is pointed. This latter characteristic, pointed leaf apex (*Pla*), is dominant and was mapped on chromosome 5 in a region from 0 cM to 13 cM. It is expressed in BIL 5.1 (Fig. 3). Another easy-to-recognise morphological trait associated with leaves was 'linear dark-green leaves' (*Ldgl*) with a leaf shape and colour similar to *L. saligna*. This trait was dominant and

Table 1 Overview of typical phenotypes of BILs and pre-BILs. Gene abbreviations are included whenever genetic inheritance is clear

BIL/line	Region in which trait is located (chromosome number and locus interval in centiMorgans)	Phenotype
3.1	C3 (0–16)	Reflexed involucre (*Ri,* finemapped at 0–3 cM)
4.2	C4 (18–61)	Linear, dark-green leaves, open head (*Ldgl*)
4.6	C4 (114–142)	Blistered leaves
5.1	C5 (0–57)	Pointed leaf apex (*Pla,* finemapped at 0–15 cM)
5.2	C5 (32–90)	Highly branching
7.3	C7 (62–75)	Early bolting (*Eb*)
8.3+8.4	C8 (76–91)	Brown seeds instead of black like those of both parents, *L. saligna* CGN 5271 and *L. sativa* Olof
9.1	C9 (0–35)	Necroses on leaves and stem (finemapped at 0–4 cM)
9.2	C9 (35–58)	Irregular and not-waxy leaf surface; irregular leaf colour distribution with many light-green areas

only expressed in BIL 4.2. *L. sativa* has a nonreflexed involucre that causes the retention of mature seeds, while *L. saligna* has a reflexed involucre at maturity that promotes seed dispersal. This trait was mapped on chromosome 3 from 0 cM to 3 cM and is expressed in BIL 3.1.

Some morphological characteristics were found in these BILs and preBILs but not in *L. saligna* or *L. sativa* Olof. For example, on the region of chromosome 9 from 0 cM to 4 cM is the character of spontaneous necroses on leaves and stems in adult plants, most severe in winter and least severe in summer. This phenotype is expressed in preBIL 9.1. Another trait that was dominant was early bolting (*Eb*), associated with an introgression of chromosome 7 in the region from 62 cM to 75 cM.

Some of the BILs showed quantitative variation between BILs for some traits, such as number of branches, leaf shape, leaf colour, leaf surface and seed colour (Table 1). The seed set of all BILs was similar to that of *L. sativa* Olof, with a few exceptions: BIL 4.2, BIL 7.2, BIL 7.3 and preBIL 9.1 had a low seed set and, in contrast, BIL 4.6 was very fertile with almost double the number of seeds per capitulum (an average of 20 seeds per capitulum) as *L. sativa* Olof (11 seeds).

Extra DNA marker information

During the development of a genetic map based on a F_2 population (Jeuken et al 2001), several AFLP markers could not be scored due to closely migrating or faint amplification products. As BILs are genetically very similar, the number of polymorphic AFLP markers between them is very limited and, consequently, these markers can be scored more unambiguously than in an F_2 population. By using BILs, we identified 269 additional clearly segregating AFLP markers that were not identified in the F_2 population even though the same AFLP primer combinations were used (Fig. 4). The total AFLP map now comprises 757 markers, which is an extension of 55% (Jeuken et al 2001). One example of a useful new BIL-marker is "E45M48-163sal", which is the only marker from the 757 markers that is mapped in preBIL 9.1 and BIL 9.2. Therefore, this marker links the two introgressions of these BILs that did not previously share overlapping markers in their introgression segments. BIL-marker E45M48-163sal can now be positioned between the F_2 markers E51M49-127 at 33 cM and E35M59-339 at 37.9 cM, previously the two outermost F_2 markers of the introgressions of preBIL 9.1 and BIL 9.2. These BILs confirm the positions of the F_2 markers, identify new BIL-markers on chromosome fragments and are useful for accurately mapping new markers in overlapping introgression fragments in BILs.

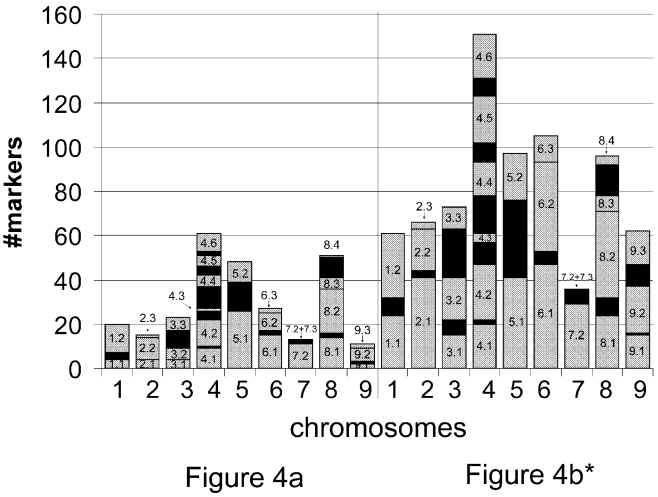

Fig. 4 **a** Number of additional BIL-markers (AFLP) that have not previously been mapped in the F_2 population (Jeuken et al 2001). **b** Number of all markers mapped on the set of 28 lines based on F_2- and BIL-markers. For both **a** and **b** the number of markers is shown per chromosome. Within each chromosome the markers are placed in subclasses of the BILs on which they are uniquely mapped. *Black areas* represent markers that were identified in the introgression interval that is shared between two BILs. *The total number of markers mapped on the set of 28 lines is 747. The genetic lettuce map comprises 757 markers. Ten markers are lacking in the BILs as they are mapped on regions that could not be introgressed in *L. sativa*. Six of these markers map on the *top* of chromosome 7 and four on the *bottom* of chromosome 3

Discussion

We have developed a set of 28 lines covering at least 96% of the *L. saligna* genome. The undesirable traits of sterility and decreased vigour that appear in F_2 populations were not manifest in the BILs, but distorted segregations did complicate development of the latter. Generally, four to five backcross generations and one selfing generation were used to obtain the BILs despite the fact that a genetic linkage map only became available after three backcross generations. Our approach was more efficient than some of the others previously used that mostly included fewer backcross generations and more selfing generations like, for example, $BC_1S_6BC_3S_1$ (Eshed and Zamir 1994), BC_2S_3 (Monforte and Tanksley 2000), BC_2S_6 (Chetelat and Meglic 2000) and BC_1S_5 (Lin et al. 1998).

The genetic linkage map that we used for MAS was accurate since the chromosome regions of the BILs confirmed the marker orders in the F_2 linkage map. The accuracy of the position of most markers on the genetic map is less than 4 cM. Those new markers that are mapped by BIL association have in general an accuracy of about 30 cM (depending on the size of the introgression).

L. saligna genome coverage in BC_1 and BC_4

The average recurrent parent genome coverage in the BC_1 and BC_4 was very similar to the expected values of 75%

and 97%, respectively, indicating that there is no general preference for alleles of one of the parents.

Between 30% and 36% of the *L. saligna* genome was lost during backcrossing without MAS from the BC_1 to BC_4 generations. Most of the lost introgressions were also not present in the BC_3 generation. This could be due to the small sampling sizes in the BC_1, BC_2 and BC_3 generations. However, the backcrosses from BC_2 to BC_3 to BC_4 were made only unidirectionally with *L. sativa* Olof as the maternal parent. This unidirectional backcrossing could have caused selection against genes affecting reproductive processes such as egg cell and pollen production or zygote fitness. Of the eight missed introgressions in the BC_4, four showed a distorted segregation with an excess of *L. sativa* alleles in the F_2 population (Jeuken et al. 2001). These introgressions were two regions on chromosome 9 (0–33 cM, 58–101 cM) and two regions on chromosome 7 (0–25 cM, 25–75 cM). This suggests that selection had taken place via genes effecting pollen fitness or zygote viability. The absence of the other introgressions, with no distorted segregation ratio in the F_2 population, could be explained by the small sample sizes. To prevent a loss of introgressions due to differences in pollen fitness, we recommend using the recurrent parent as a male parent. Another way to prevent losing introgressions would be to use MAS during the backcross programme in each generation from BC_1 till BC_4. In that way a missing introgression would be noticed immediately, and it would require little effort to retrieve it from an earlier generation. We conclude, therefore, that the best solution is to use MAS and the recurrent parent as the pollen donor.

L. saligna genome coverage of a set of BILs

Our set of 28 lines covers at least 96% of the *L. saligna* genome; only 3% of the *L. saligna* genome was lost. The absence of some portion of the genome in a final set of BILs has also been observed during the development of other sets of BILs and has been ascribed to lethal combinations of genes from the two donor parents (Chetelat and Meglic 2000; Monforte and Tanksley 2000). At least 77% of the *L. saligna* genome is present in 24 lines that are completely homozygous (BILs and doubleBILs). This homozygosity is a very valuable prerequisite for the quality of trait analyses in future research as every phenotypic difference between the BILs can immediately be associated with the unique introgressed segment from *L. saligna*. The other lines segregating for an introgression from *L. saligna* will also show segregation of associated traits. Overall, these 28 lines are very valuable lines to use in the study of all the traits from *L. saligna* as they profit from all the benefits of BILs described in the Introduction.

The 20 BILs with one homozygous introgression contained on average 4% (33 cM) of the *L. saligna* genome, which falls within the expected range of 6% (1/16) and 3% (1/32), based on four to five backcrosses and one selfing.

Distorted segregation ratio

In large segregating populations of backcross lines and selfing lines distorted segregations have been observed with preferences for both *L. saligna* and *L. sativa* alleles. Again there is no evidence for a general preference for alleles of one of the parents. This is in contrast with some studies in BIL development (Monforte and Tanksley 2000, Chetelat and Meglic 2000), in which a deficit of the wild allele has been observed. Surprisingly, distorted segregation ratios were also observed for chromosome regions that did not show any segregation distortion in the F_2 population (Jeuken et al 2001). The most likely explanation is a lethal combination of genes from the two donor parents in the preBIL. This lethal combination of genes seems to be compensated in F_2 plants by the presence of other genes absent in the preBIL.

Two examples of skewed segregation ratios only after backcrosses with the backcross line as the paternal parent and not as the maternal parent were observed. In one case the skewness showed a preference for *L. sativa* alleles, which is likely explained by reduced pollen or zygote fitness. The other example showed a preference for *L. saligna* alleles, which is likely explained by increased pollen or zygote fitness. In both cases it is not known whether the presence of a gene of *L. saligna* or the absence of a gene of *L. sativa* is responsible.

Morphological characteristics

Pointed leaf apex (*Pla*) was identified in the backcross lines, in progeny from selfed lines and in BIL 5.1. As this trait was associated with homozygous and heterozygous introgressions, it was dominant. It was probably monogenic or otherwise explained by closely linked genes within a map distance of 13 cM. This is in agreement with asparagus lettuce, in which a single dominant gene has been found expressing pointed leaf apex (Lindqvist 1960). This morphological trait can be used as an easy scorable marker as it was easily recognisable at an early stage of plant development.

Reflexed involucre (*Ri*) was also identified in lines with the specific introgression in heterozygous or homozygous state. Therefore, this trait was also dominant and probably monogenic. Lindqvist also found that the *L. saligna* allele for this trait was dominant over the *L. sativa* allele in F_1 plants (Lindqvist 1956; Robinson et al. 1983). Reflexed involucre is easy to score, but only at a very late plant stage when seed is maturing. This makes it less valuable as a morphological marker. The typical phenotypes of most other BILs can also be used as morphological markers. Up to the time of this investigation these traits had not been located.

Perspectives of BILs

This study demonstrates that BILs can be used for mapping simple morphological traits and for identifying and mapping region-specific molecular markers. Other perspectives with a high potential for the lettuce BILs are mapping expressed sequence tags of *L. sativa* and *L. saligna* and the genetic dissection of agriculturally valuable traits including quantitative traits with complex inheritance patterns like the *Bremia* resistance of *L. saligna.* These quantitative traits are usually difficult to study in other population types.

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