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## A detailed linkage map of lettuce based on SSAP, AFLP and NBS markers

Received: 15 July 2005 / Accepted: 13 November 2005 / Published online: 10 December 2005  
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**Abstract** Molecular markers based upon a novel lettuce LTR retrotransposon and the nucleotide binding site-leucine-rich repeat (NBS-LRR) family of disease resistance-associated genes have been combined with AFLP markers to generate a 458 locus genetic linkage map for lettuce. A total of 187 retrotransposon-specific SSAP markers, 29 NBS-LRR markers and 242 AFLP markers were mapped in an F<sub>2</sub> population, derived from an interspecific cross between a *Lactuca sativa* cultivar commonly used in Europe and a wild *Lactuca serriola* isolate from Northern Europe. The cross has been designed to aid efforts to assess gene flow from cultivated lettuce into the wild in the perspective of genetic modification biosafety. The markers were mapped in nine major and one minor linkage groups spanning 1,266.1 cM, with an average distance of 2.8 cM between adjacent mapped markers. The markers are well distributed throughout the lettuce genome, with limited clustering of different marker types. Seventy-seven of the AFLP markers have been mapped previously and cross-comparison shows that

the map from this study corresponds well with the previous linkage map.

### Introduction

Molecular markers have revolutionized genetic analysis of crop plants, where they play an important role in linkage analysis, physical mapping, quantitative trait loci (QTL) analysis, marker-assisted selection and map-based cloning (Bernatsky and Tanksley 1989; Lande and Thompson 1990; Knapp 1998). Genetic linkage maps based on a variety of molecular markers have become important tools for the analysis of plant genomes and aid in various plant breeding and genome analyses activities (Jeuken et al. 2001). Accurate, high-resolution genetic maps are very important tools to locate the genes encoding desirable traits. Once flanking markers affecting a particular trait are identified, marker-assisted selection can be performed at the DNA level to accelerate the improvement of crop plants or livestock.

The efficiency of marker-assisted genetic analysis has been greatly increased with the availability of various kinds of dominant and co-dominant markers such as AFLP, Sequence-specific amplification polymorphisms (SSAP), SSRs and nucleotide binding site (NBS) profiling etc. (Vos et al. 1995; Waugh et al. 1997; Mei et al. 2004; van der Linden et al. 2004; Syed et al. 2005). The usefulness of any given marker system depends on the species under study and the chosen application. SSR (microsatellite) markers are powerful because they are co-dominant in nature, multi-allelic and hence more informative, compared to these other marker types. However, they require significant input for their discovery and their hypermutability brings disadvantages in situations where wide germplasm is under analysis because of the possibility of homoplasy.

For crops where there is little or no DNA sequence information available, anonymous multiplex marker systems, such as AFLP and its derivatives have been favored (Vos et al. 1995; Sebastian et al. 2000; Jeuken

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Communicated by J. S. Heslop-Harrison

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et al. 2001). AFLP is a PCR-derived method that reveals multiple restriction fragment length polymorphisms as bands on a gel, without the need for any sequence information of the DNA under study. In this way hundreds of anonymous molecular markers can be generated rapidly in any species. AFLP markers have been extensively used for genetic mapping and QTL analysis in different species (e.g. Castiglioni et al. 1999; Klein et al. 2000; Sebastian et al. 2000; Jeuken et al. 2001). The efficiency of AFLP markers has been further enhanced by the development of co-dominant scoring software by Keygene but this has increased the expense of the method.

Sequence-specific amplification polymorphisms marker technology (Waugh et al. 1997) was developed from AFLP (Vos et al. 1995) and anchored PCR applied to transposable elements (Korswagen et al. 1996). SSAP is an anchored AFLP approach that uses a locus-specific, labeled primer, together with an adapter-specific primer to convert the gap between any locus-specific DNA sequence and a flanking restriction site into a gel band. SSAP was initially developed using long terminal repeat (LTR) retrotransposon insertions as loci (Waugh et al. 1997; Ellis et al. 1998). SSAP has been applied to barley, pea, wheat and alfalfa. In these studies SSAP markers revealed twofold to threefold higher polymorphism rates per primer combination than AFLP markers (Waugh et al. 1997; Ellis et al. 1998; Flavell et al. 1998; Gribbon et al. 1999; Porceddu et al. 2002; Queen et al. 2004). The transposon display approach, which uses another transposon type called miniature inverted repeat transposable elements (MITEs) is virtually identical to SSAP and has been deployed in Maize (Casa et al. 2000).

Anchored AFLP has also been applied to specifically target resistance genes (R genes) of the nucleotide binding site-leucine-rich repeat (NBS-LRR) class (Van der Linden et al. 2004). This approach, called NBS profiling, offers the potential advantage that the markers are automatically linked to potentially useful 'functional' genes. The NBS-LRR family represents by far the largest class of currently identified plant R genes, many of which are exploited intensively in breeding practices (Van der Linden et al. 2004). Because of the high level of conservation of the NBS domain between species, degenerate locus-specific primers can be used, with no other sequence information needed. NBS profiling resembles SSAP, in that it also anchors a PCR reaction to a specific genomic sequence with a motif-specific primer and an adapter primer. However, it is not based on the protocol and adapters utilized by AFLP. NBS profiling has been successfully applied for mapping of resistance gene analogues (Calenge et al. 2005) and biodiversity studies (Reeves et al. 2004; Mantovani et al. 2004).

Different marker types target different genomic regions and show different distribution patterns (Klein et al. 2000; Sebastian et al. 2000; Mei et al. 2004; van der Linden et al. 2004). Therefore, a diversity of different molecular markers is preferable for a versatile molecular marker map. To construct a detailed linkage map of

lettuce we have employed the three technically similar but diverse techniques of AFLP, SSAP and NBS profiling. The markers have been revealed in an interspecific cross between a *Lactuca sativa* cultivar commonly used in Europe and a wild *Lactuca serriola* isolate from Northern Europe, with the goal of using the markers to assess gene flow from cultivated lettuce into the wild, as a model for genetically modified (GM) crop release (Van de Wiel et al. 2003), a subject that has gained substantial public and scientific concern over the last decade (Hails 2000; Gray 2004; Snow et al. 2005). This has also given us the opportunity to compare these three marker types for their accessibility and genomic distribution in this species.

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## Materials and methods

### Plant materials and DNAs

An  $F_2$  population of 90 individuals was generated from an interspecific cross of *Lactuca serriola* DH\_M21(SER) (P1)  $\times$  *L. sativa* cv Dynamite (Nunhems zaden, 01-2002, 60826) (P2). DH\_M21(SER) was collected from the wild in Eys, The Netherlands in 2001 (Hooftman et al. 2005). The interspecific hybridisation followed standard protocols as described by Nagata (1992) and Ryder (1999). DNAs were extracted from lettuce seedlings by a modified CTAB extraction method (Saghai-Marooof et al. 1984; Virk et al. 1999).

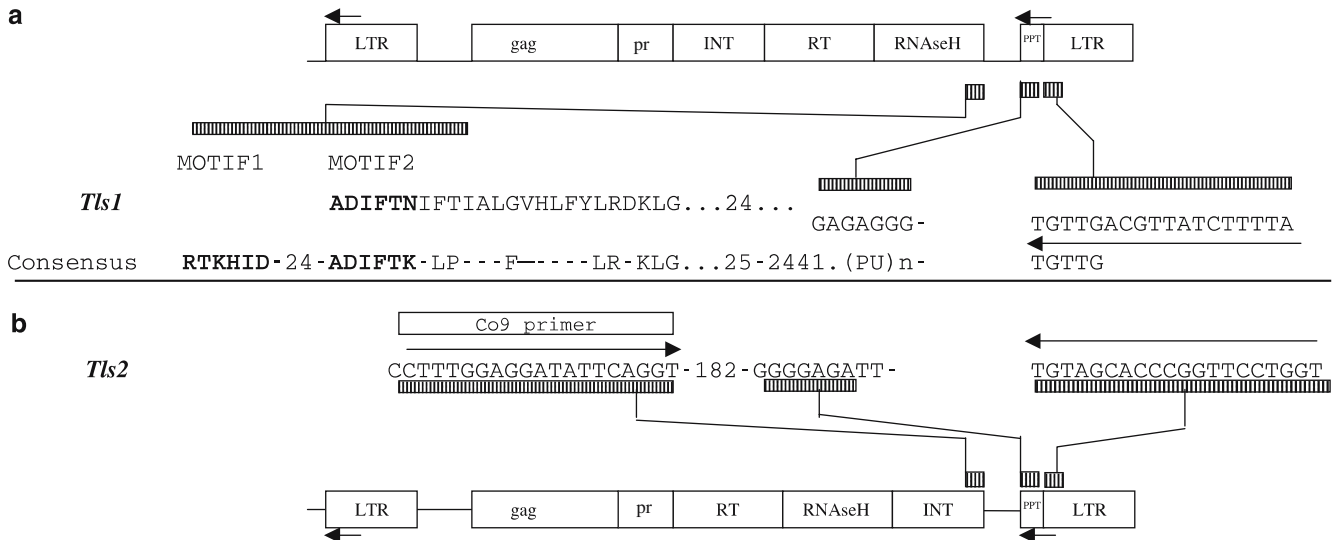
### Isolation and characterization of retrotransposon LTR sequences from lettuce

#### (i) LTR characterisation for Ty1-copia group retrotransposons

For isolating lettuce LTR sequences from Ty1-copia group retrotransposons, the protocol of Syed et al. (2005), a modification of the method of Pearce et al. (1999), was used. This is basically the SSAP approach, with modifications, using two nested degenerate primers to PCR amplify unidirectionally outwards from a conserved protein-coding region in the retrotransposon interior towards the LTR. The LTR-interior junction sequence is recognized by the presence of conserved motifs (Fig. 1a).

#### (i) LTR characterisation for Ty3-gypsy group retrotransposons

Long terminal repeat sequence from the Ty3-gypsy group retrotransposon *Tls2* was obtained by the same basic methodology as used for Ty1-copia group retrotransposons, with the difference that the start point for the walk outwards from the retrotransposon interior was derived from the QGB4co9 EST, whose sequence



**Fig. 1** Structural features of Ty1-*copia* and Ty3-*gypsy* group retrotransposons and identification of LTR sequences for *Tls1* and *Tls2*. The *RNaseH*-PPT-LTR junction sequence of *Tls1* obtained in this study is compared with the consensus region for Ty1-*copia* retrotransposons (not to scale; adapted from Pearce et al. 1999). The sequence of *Tls2* is also shown, showing the

different arrangement of genes for Ty3-*gypsy* group retrotransposons (*int*-PPT-LTR). The sequences and polarities of the primers for the SSAP experiments are shown with *arrows* on the diagram and *below* the sequences themselves. The SSAP marker method amplifies fragments extending leftwards from the *leftmost arrows* in the figure

comes from the integrase gene of a Ty3-*gypsy* group retrotransposon near the interior-LTR junction (EMBL accession BQ856448). The LTR-interior junction was identified from the walk sequence in the same way as described earlier (Fig. 1b).

#### AFLP molecular marker analysis

AFLP analysis was performed according to Vos et al. (1995). The primer combinations used for the mapping were selected based on a prescreening of the parent individuals and the estimation of genome coverage, based on the information of the proprietary Keygene integrated lettuce genetic linkage map (unpublished). 6 *EcoRI/MseI* and 6 KeyGene proprietary primer combinations (PCs) were used (Table 1). All AFLP markers

were scored as co-dominant by using the proprietary software developed by KeyGene.

#### SSAP molecular marker analysis

The SSAP procedure was performed exactly as described by Syed et al. (2005), a modification of Waugh et al. (1997). The Ty1-*copia* group retrotransposon *Tls1*-specific primer isolated by the gene-walker approach was TAAAAGATAACGTCAACACA, plus T, A or G selective base. The *Tls2* Ty3-*gypsy* group retrotransposon primer was ACCAGGAACCGGGTGCTACA plus T, C or G selective base. The selective bases on the retrotransposon-specific primers were chosen to introduce mismatches with the bases immediately preceding the corresponding 3' LTRs, thus inhibiting the production of

**Table 1** Primer combinations for AFLP and SSAP markers are shown with the number of polymorphic markers generated for each of them

AFLP		SSAP			
Primer combination	No. of markers	Primer combination	No. of markers	Primer combination	No. of markers
E35/M48	28	GTG	7	AAG	8
E35/M49	21	TCT	18	ACG	9
E35/M59	37	CTA	14	ACT	15
E44/M48	23	AAT	17	ATA	9
E44/M49	22	AAC	13	ATC	10
E45/M48	30	ATG	13	ATT	10
1A39	18	GTT	16	CTC	17
1A36	23	CTG	17	CTT	11
1A40	12	TCC	13		
1A37	15	ACA	19		
1A38	13				
1A31	20				
<b>Total</b>	<b>262</b>		<b>147</b>		<b>89</b>

SSAP products from the 3' LTRs of *Tls1* and *Tls2*, which show no polymorphism and are unmappable, because they represent multiple retrotransposon internal sequences. For the F<sub>2</sub> mapping study three selective bases were also used on the adapter primer to reduce the gel band number to a manageable level.

### NBS profiling

NBS profiling was performed as described by Van der Linden et al. (2004), with modifications. In brief, ca. 200 ng of genomic DNA was digested with restriction enzyme *Mse I*, and the adapter was ligated to the fragment ends in a single reaction in 5×RL+ buffer (Vos et al. 1995), 1 mM ATP, 10 U *Mse I*, and 1 U T4 DNA ligase in 60 µl reaction volume. Next, 2.5 µl of the RL mixture was used as a template in PCR with 20 pmol of NBS-specific primer, 20 pmol of adapter primer, 0.25 mM dNTPs, 0.4 U HotStar Taq DNA polymerase (Qiagen), 2.5 µl 10× PCR buffer in a total volume of 25 µl. PCR conditions were an initial 15 min at 95°C, and 30 cycles of [0.5 min 95°C, 1.4 min 55°C (primer NBS5a; YYTKRTHGTMITKGATGAYGTITGG) or 60°C (primer NBS3; GTWGYTTICCYRAICCISSC-ATICC), 2 min 72°C]. The mixture was diluted 10 times with distilled water, and 5 µl was used as a template in a second PCR with a  $\gamma^{33}\text{P}$ -ATP labeled NBS-specific primer (0.5 pmol), 2 pmol of adapter primer, 0.25 mM dNTPs, 0.4 U HotStar Taq DNA polymerase, 2.5 µl 10× PCR buffer in a total volume of 25 µl. Samples were electrophoresed on 6% polyacrylamide sequencing gels and visualized by autoradiography.

### Genetic linkage mapping

Genetic mapping of the scored markers in the F<sub>2</sub> of *L. sativa* (DH\_M21(SER)) × *L. serriola* population was carried out by using JoinMap 2.0 (Stam and van Ooijen 1995). For the F<sub>2</sub> segregation ratios, a test for skewness was performed, with a 0.5% threshold level for significance. AFLP markers scored co-dominantly were tested against the predicted 1:2:1 ratio (corresponding to homozygous *L. sativa*:heterozygotes:homozygous *L. serriola*). Dominantly scored SSAP and NBS markers were tested against the predicted 3:1 ratio, representing [homozygote + heterozygote]:[homozygotes] by marker band presence:absence, respectively. Markers were assigned to linkage groups (LGs) by increasing the LOD score for grouping with steps of one LOD unit. Mapping was carried out with the following thresholds; REC of 0.45, LOD of 0.01, JUMP of 4 and TRIPLET of 7. No order was forced during the linkage analysis. Recombination frequencies were converted to map distances in centimorgans (cM) using the Kosambi mapping function (Kosambi 1943) and the genetic map was drawn using the MapChart program (Voorrips 2002).

## Results

### Isolation of LTR sequences and SSAP marker development in lettuce

No prior DNA information is needed for the application of AFLP markers in lettuce but the development of SSAP in this species requires sequence information for the terminal regions of the retrotransposon (Vaugh et al. 1997). The LTR sequences are present at the ends of LTR retrotransposons and consist of identical direct repeats (Fig. 1). Unfortunately, the LTR sequences cannot be predicted as they do not contain any conserved motifs and they are usually too long to allow SSAP from regions internal to them (Vaugh et al. 1997). Therefore, to obtain LTR sequence information, SSAP is performed outwards from conserved genic regions in the retrotransposon interior (Pearce et al. 1999; Syed et al. 2005). The interior-LTR junction can often be recognized quite easily by the presence of a short polypurine tract (PPT) followed by a consensus TGTRG motif, which represents the end of the LTR (Pearce et al. 1999). The LTR sequence thus obtained from one end of the retrotransposon can then be used to design SSAP primers facing outwards from the LTR at the other end.

Using the previous approach one convincing LTR sequence was obtained for a Ty1-copia group retrotransposon (Fig. 1a), which we have termed Transposon, *Lactuca sativa* (*Tls1*). For the Ty1-copia group, directional amplification proceeds outwards from two closely spaced, highly conserved motifs in the RNaseH gene, using a nested pair of degenerate PCR primers (Pearce et al. 1999). The RNaseH open reading frame, containing several conserved amino acid residues, continues for 18 codons before a stop codon, which is followed by a spacer region, and then the PPT follows and the TGTTG motif, characteristic of the LTR end (Fig. 1a).

To isolate an LTR from the gypsy subgroup of LTR retrotransposons a different approach is necessary, because no well-conserved motifs for SSAP are adjacent to the LTRs. Fortunately, an EST clone (Co9 in Fig. 1b) containing C-terminal sequence from the integrase gene of Ty3-gypsy group retrotransposons was generously provided to us by A. Kozic and R. Micheltore. This sequence was used as a start point to walk outwards from the *int* gene in the direction of the LTR (Fig. 1b). Again, the presence of a PPT followed by TGTT motif characterized the end of the Ty3-gypsy group retrotransposon LTR, which we have called *Tls2*.

Sequence-specific amplification polymorphisms primers specific for the LTRs of *Tls1* and *Tls2* were designed to amplify DNA in an upstream direction from the 5' LTRs of corresponding retrotransposon insertions (Fig. 1). To optimize the SSAP protocol for lettuce the two parents of our F<sub>2</sub> mapping population were used (*L. serriola* and *L. sativa*; Fig. 2). The quality of banding patterns produced by *Tls2* with



various primer combinations was significantly superior to that of *Tls1*, so the former was used exclusively in subsequent analyses. Using 18 primer combinations, a total of 236 polymorphic SSAP bands were scored in

the  $F_2$  population, an average of ca. 13 markers per primer combination. An example is shown in Fig. 3a.

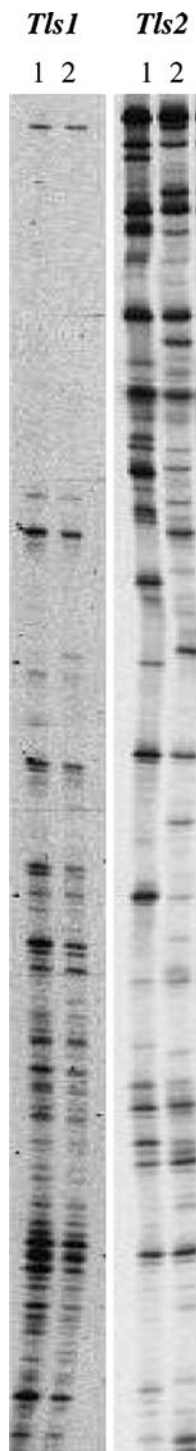
#### NBS-profiling marker development in Lettuce

Nucleotide binding site-profiling has been developed to work with similar primers and enzymes in a number of crops without modifications (Van der Linden et al. 2004). On the basis of available sequences for *Lactuca* resistance gene analogue sequences, we decided to use the NBS3 primer, which is virtually identical to the NBS2 primer of Van der Linden et al. (2004) and NBS5 primer as used by the same workers. DNA was digested with *Mse*I. Trial experiments with samples run in duplicate showed that the procedure is highly reproducible in lettuce (Fig. 3). Each of the profiles generated between 60 and 90 bands, of which about half were polymorphic within a *L. sativa* cultivar set, and more were polymorphic between species (Fig. 3). This validated marker system of NBS profiling was then used to genotype the  $F_2$  genetic mapping population.

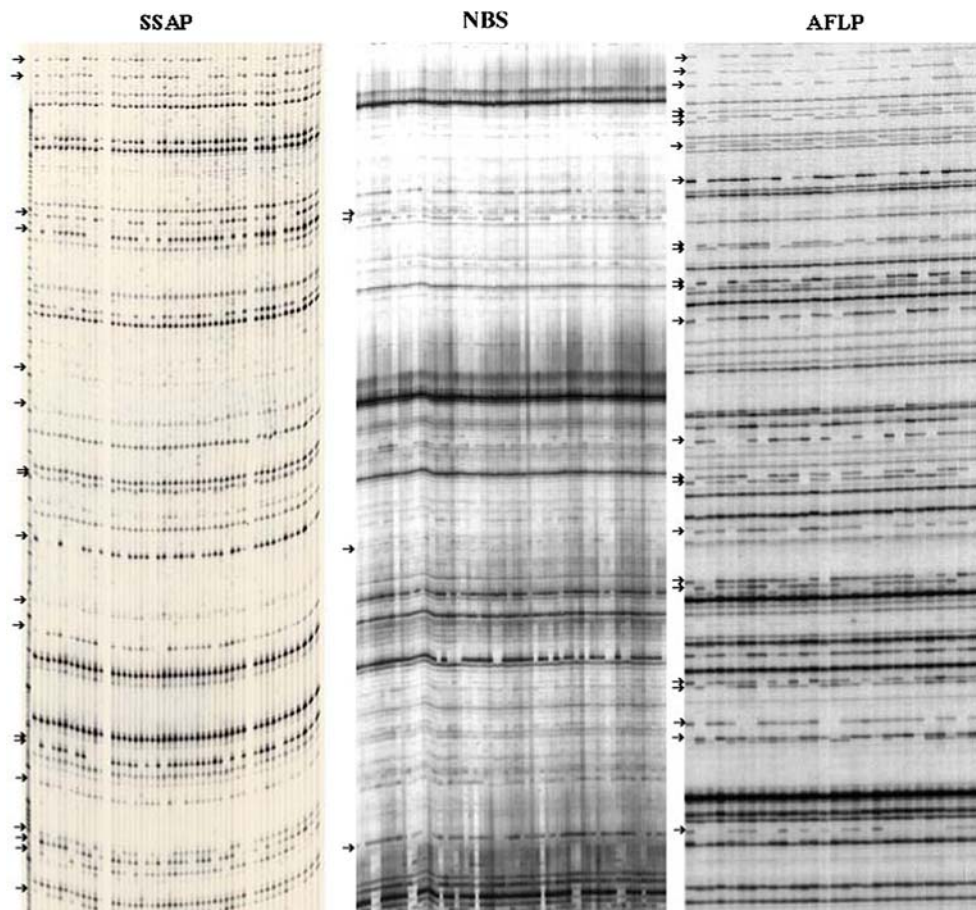
To get an indication of the resistance gene analogue (RGA) content in lettuce, a number of bands from each profile were sequenced. For the NBS5 primer, several bands could be positively identified as putative RGAs (Van der Linden et al. 2004; our unpublished results). These included RGC2 candidates, of which one has been identified as the *Dm3* gene (Meyers et al. 1998). A substantial number of bands (more than 50%) had no significant similarity to any of the sequences in the database (using standard settings for the XBLAST and the NBLAST programs).

#### Linkage analysis in lettuce

The mapping population chosen for this study was designed to contain segregating markers, which could be of use in the study of introgression of cultivated lettuce genetic material into wild germplasm. The population was generated initially from an interspecific  $F_1$ -cross between *L. serriola* (P1) and *L. sativa* (P2). For the latter, we used a common European cultivar (cv. Dynamite: Nunhems zaden, 01-2002, 60826: purchased April 2002). This line possesses genetic material from another wild relative (*L. virosa*) and may possess genetic material originally derived from *L. serriola*. The *L. serriola* parent lineage [DHM21(ser)] comes from the Netherlands, it forms part of the University of Amsterdam's *L. serriola* seed collection and was sampled in 2001. DHM21(ser) was derived from a road verge population in Eys, province of Limburg, within the core of the historic (nineteenth and early twentieth century) *L. serriola* distribution. This large population is known to fluctuate between 1,000 and 100,000 or more reproductive plants (Hooftman et al. 2005). Furthermore, no private gardens exist in its close vicinity, which could be the source of recent contaminating genes from *L. sativa*.



**Fig. 2** SSAP marker profiles of *Tls1* and *Tls2*. Profiles are shown for the two parents of the mapping population; *Lactuca serriola* (1) and *Lactuca sativa* (2). *Tls1* and *Tls2* retrotransposon-specific primers had A and T selective bases, respectively, at their 3' ends and, in both cases, CA on the *Mse* I adapter primer



**Fig. 3** SSAP, NBS and AFLP marker profiles in a segregating lettuce  $F_2$  population. Markers used for this study are *arrowed*. Primer combinations for SSAP and NBS profiling were *Tls2*+TCT and *NBS5a/MseI*, respectively

The 187 SSAP and 29 NBS markers described previously were complemented with 242 newly generated AFLP markers, to allow cross-comparison between the three marker methods and to increase the diversity of marker types represented (see [Introduction](#)). Altogether, 458 of the 536 markers were assigned to nine major and one minor linkage group (LG) at a logarithm of the odds (LOD) threshold of 5 (Fig. 4). The 458 markers gave a total map length of 1,266.1 cM and an average genetic distance between adjacent mapped loci as 2.76 cM (Fig. 4). The genetic map generated here also corresponds well with the integrated genetic linkage map of Keygene, which is derived from the map of Jeuken et al. (2001) (data not shown). LGs 1–9 in our map correspond to LGs 1–9 in the Jeuken et al. map. The status of LG 10 is unclear at present (lettuce has nine chromosomes). Altogether, LGs 1–9 of the integrated map contain 5, 8, 10, 8, 10, 6, 13, 8 and 9 AFLP markers, respectively, from the  $F_2$  map described here. These bridging markers show similar distributions on the two linkage maps. However, a few differences are apparent, which could be due to mobility error, co-migration of bands and/or rounding errors of marker names.

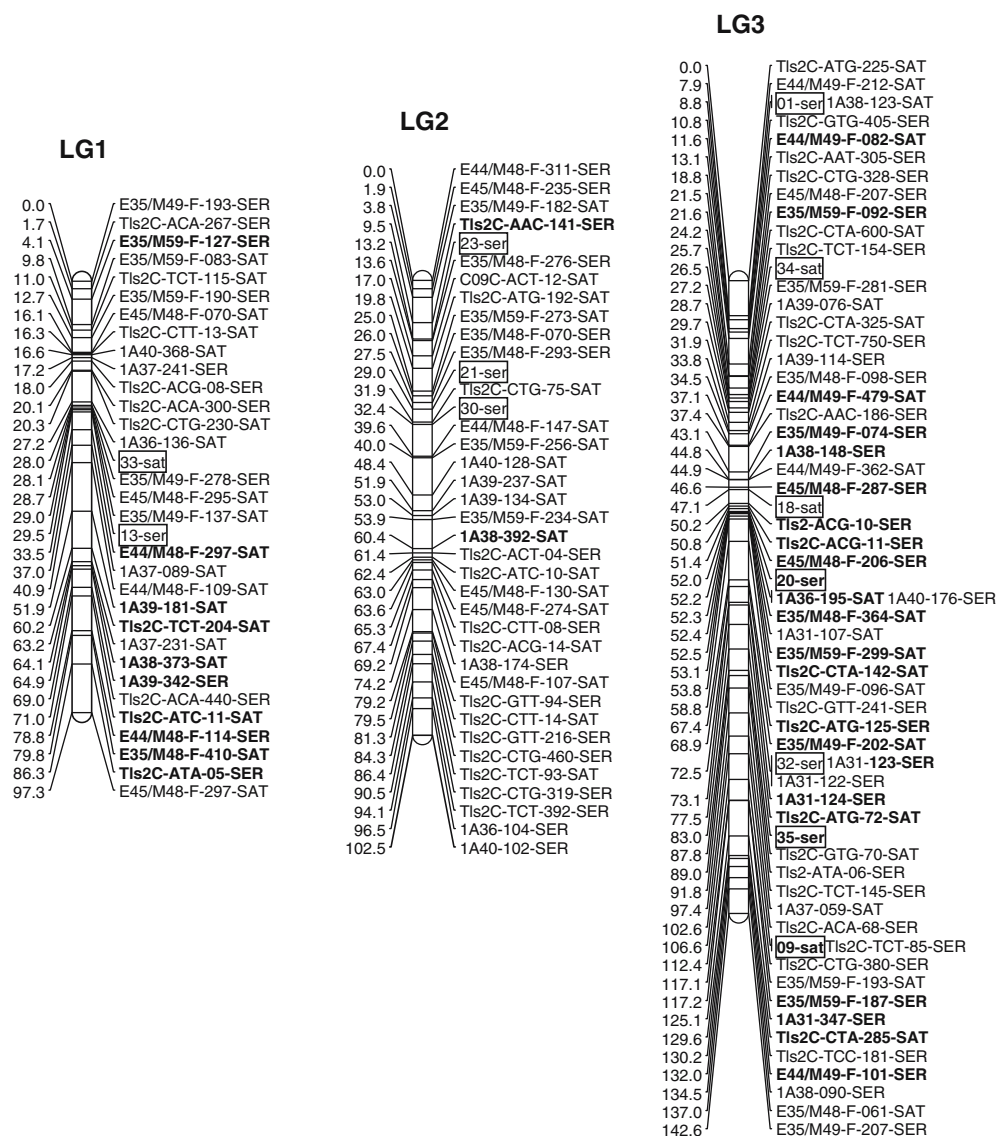
The length of a previously published map based on RFLP and RAPD markers was 1,950 cM for 13 major

and 4 minor linkage groups (Kesseli et al. 1994). AFLP markers on our map of 1,266.1 cM correspond well to the 854 cM map of Jeuken et al. (2001). The addition of SSAP and NBS markers has contributed to an increase in the overall map length of our map but it is still much smaller than the map of Kesseli et al. (1994), which was based on 66  $F_2$  individuals, and RAPD markers (41% of the map), which are not considered to be a totally reproducible marker class (Jeuken et al. 2001). The proportions of each marker type that failed to map were 8, 20 and 23% for AFLP, SSAP and NBS, respectively. Aside from the single small linkage group, the map corresponds well with the nine chromosomes of lettuce.

#### Marker distribution

All the markers appear to be quite randomly distributed along all LGs, except LGs 7 and 8, which show rather few markers in the lower parts of these linkage groups. If the AFLP, SSAP and NBS markers were evenly distributed across the LGs, they should be present on each at a ratio of 0.52:0.40:0.08, respectively. In total, (21, 10, 2); (20, 15, 3); (33, 23, 7); (25, 24, 2); (42, 38, 5); (13, 14, 1); (31, 22, 3); (22, 17, 2); (28, 22, 3); (7, 2, 1) AFLP,

**Fig. 4** Linkage map for an F<sub>2</sub> population derived from a cross between *L. sativa* × *L. serriola*. Markers shown in **bold** exhibit skewed segregation, and SER or SAT designations refer to the origin of alleles from *L. serriola* or *L. sativa*, respectively. NBS markers are **boxed**, SSAP markers are prefixed by Tls2 and AFLP markers by E or 1A (see Table 1)



SSAP and NBS markers were mapped on LGs 1–10, respectively. These ratios are indeed very close to the expected values, based on the chi square test, except LG 1. However, even for LG 1, the chi square test failed to pick any statistically significant difference between the markers (data not shown). As mentioned above, 77 AFLP markers could be identified on the Keygene integrated map, developed from that of Jeuken et al. (2001), and in general the locations agree between the two maps.

#### Segregation distortion of genetic loci

For the majority of the marker loci mapped here (ca. 81%) the observed genotypic frequencies do not differ significantly from the expected segregation ratios of 1:2:1 for co-dominant AFLP and 3:1 for dominant SSAP and NBS-LRR markers, respectively. Segregation distortion based on the chi square test (data not shown)

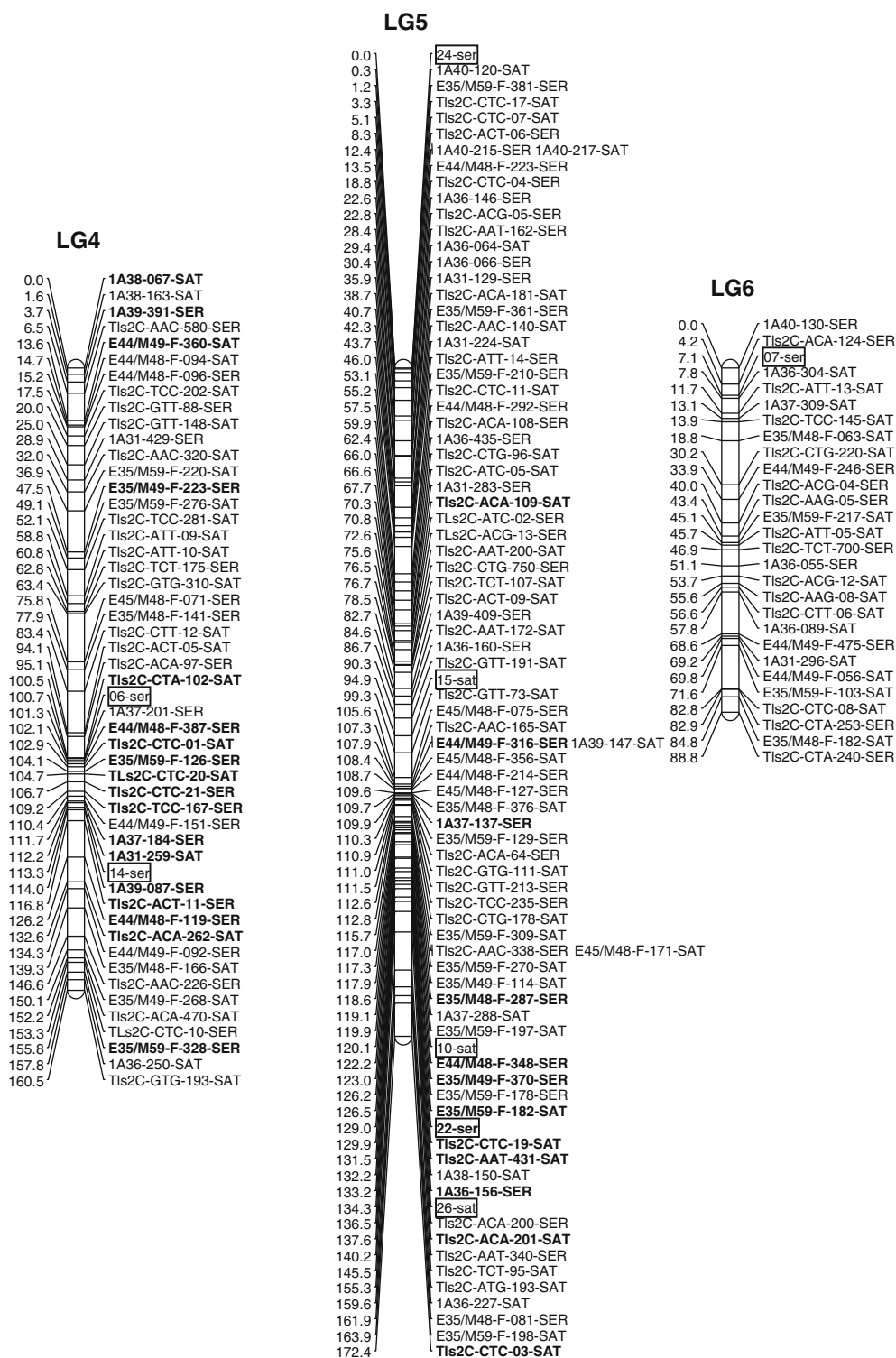
was significant for 88 loci (ca. 19% of the markers), including 59 AFLP, 24 SSAP and 5 NBS loci, mapped in various LGs (shown in bold font markers on the map in Fig. 4). Roughly half of these cluster into four regions of LGs 1, 3, 4 and 5, respectively. The rest are quite randomly distributed throughout the genome. LG3 shows the maximum number of markers deviating from normal ratios, whereas LG4 shows a cluster of markers with segregation distortion in the lower part of the map. The abnormal allele ratios were evenly distributed between the two parental chromosome sets (data not shown).

#### Discussion

In this study, we have used three complementary marker technologies to generate a genetic linkage map for lettuce. The application of the SSAP approach has required the isolation of LTR sequence information from retrotransposons of lettuce and two novel lettuce



Fig. 4 (Contd.)



LTRs have been isolated, one corresponding to a *Ty1-copia* group LTR retrotransposon and the other to a *Ty3-gypsy* group retrotransposon. The SSAP and NBS profiling molecular marker methods have been validated for lettuce and the 216 markers generated using these methods have been complemented by a further 242 AFLP markers, giving a reasonably high resolution

genetic map for lettuce, which has allowed us to compare the performances of the three marker types.

The two LTRs isolated in this study produced useful marker systems, but *Tls2* marker profiles were of higher reproducibility and polymorphism level (Fig. 2), so these have been used exclusively in this study. Judging from the number of selective bases needed to deduce the SSAP



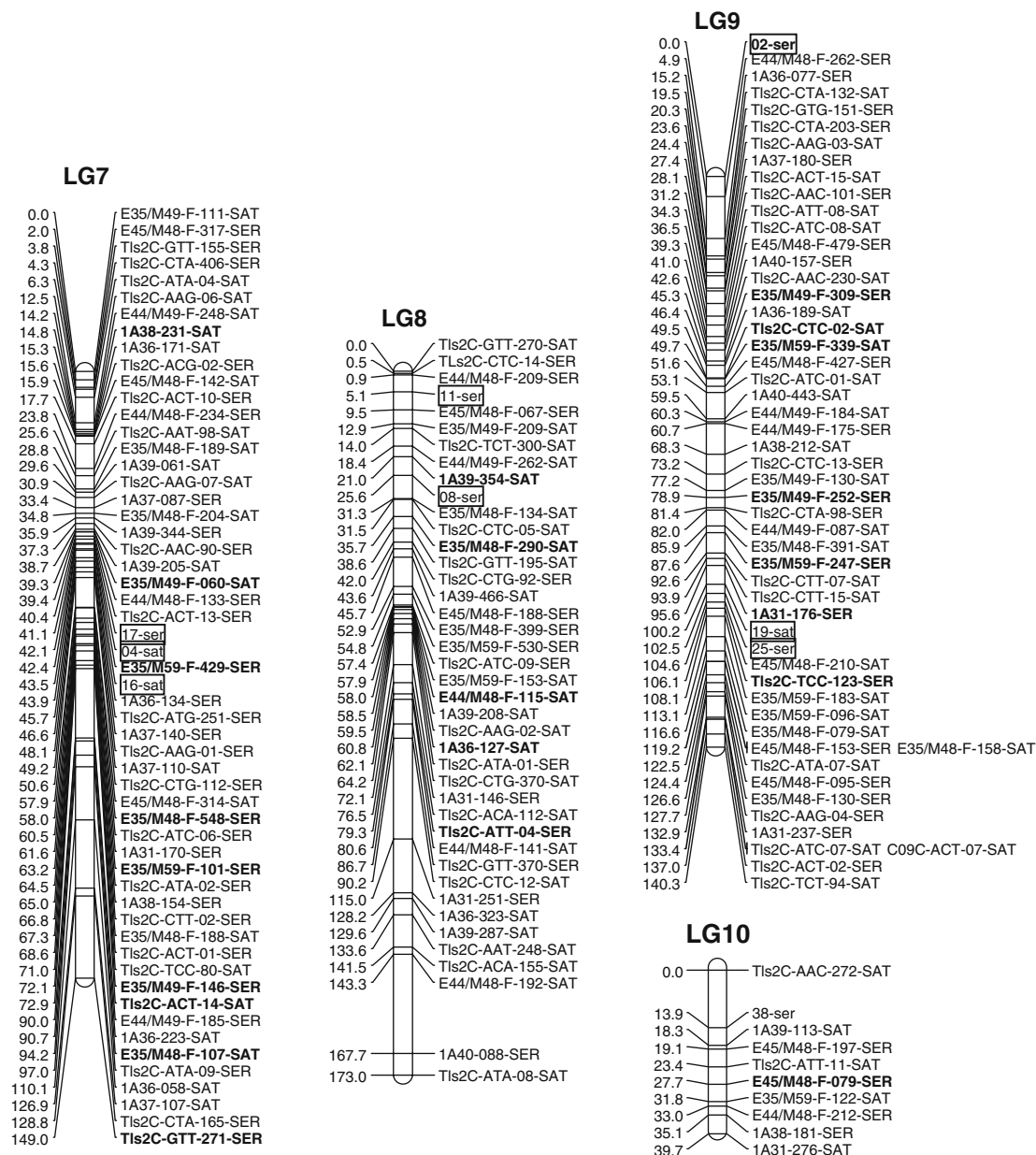


Fig. 4 (Contd.)

band number to a manageable number the copy numbers of these transposons are quite high (~800–1,000). Their broad genomic distribution (Fig. 4) is consistent with this. The distribution of *Tls2* SSAP markers suggests that it has no pronounced preference for inserting into particular genomic regions. This observation further proves the usefulness of SSAP markers for linkage mapping and their subsequent use in various genome analysis activities as explained elsewhere in this article.

Fewer NBS profiling markers were obtained than the other two marker types. This is an inherent property of such markers, as each in principle corresponds to a member of the NBS-LRR resistance gene analogue gene family that is present in far lower numbers in the plant genome than retrotransposons (SSAP) or restriction

sites (AFLP). The usefulness of NBS-LRR markers is their close linkage to potentially important resistance genes. This is likely to be an important factor in the survival of genomic segments transferred between *L. sativa* and *L. serriola* and is thus highly relevant to gene flow studies in these species. The well-studied *Dm3* NBS-LRR resistance gene, which confers resistance to infection by the downy mildew fungus *Bremia lactucae*, (Meyers et al. 1998), is located around 25 cM on chromosome 2. NBS markers 21-ser or 23-ser map to this approximate location and may derive from RGAs in a closely linked gene cluster. Sequence analysis of the NBS-LRR bands shows that less than 50% correspond to identifiable RGAs (data not shown). This may be due to the relative scarcity of RGA sequences from members

of the *Asteraceae* in the databases. Thus, the sequences which were not identified might actually be RGAs, but their sequence similarity to the relatively few known RGAs is too low to allow positive identification. However, we cannot exclude the possibility that a sizeable fraction of the NBS-LRR markers described here in fact derive from other sequence types.

The detailed linkage map of lettuce, which we have developed spans 1,266 cM, with an average distance of 2.76 cM between adjacent mapped markers. 32% of the AFLP markers are also found on the integrated linkage map of lettuce (Jeuken et al. 2001), allowing accurate cross-referencing between the two maps and giving us the opportunity in the future to exploit the much larger number of markers in the integrated map. The inclusion of SSAP markers in our map further provides efficient, stable and extensive coverage of the lettuce genome, because inserted copies of retrotransposons do not excise from their sites, unlike DNA transposons (Schulman et al. 2004). The usefulness of SSAP for studying genetic diversity, mapping populations and species relationships has been reported for barley (Vaughan et al. 1997; Leigh et al. 2003; Schulman et al. 2004), pea (Ellis et al. 1998; Pearce et al. 2000), wheat (Gribbon et al. 1999; Queen et al. 2004), oat (Yu and Wise 2000) and alfalfa (Porceddu et al. 2002). This property of genomic stability will give the map shown here an added value.

Some published AFLP linkage maps show clustering of these markers in centromeric regions, due to an excess of repeats in this area and suppressed recombination shrinking the genetic map relative to the DNA content (Jeuken et al. 2001), (Qi et al. 1998; Haanstra et al. 1999; Vuylsteke et al. 1999; Young et al. 1999). Only a small amount of overall clustering was observed in our map and this is confined to LGs 1, 3, 5, 7 and 8 (Fig. 4). Two sets of closely linked NBS markers are also apparent on LGs 7 and 9. These may represent the sites of NBS-LRR RGA clusters. The RGA cluster containing the important *Dm3* fungal resistance gene is located at ca. 25 cM on LG2. This might correspond with NBS marker 23-ser or 21-ser (13.2 and 29.0 cM, respectively on our map).

The diverse molecular markers and the linkage map described here will be useful both to the analysis of gene flow between cultivated and wild forms of the *Lactuca* genus and to the lettuce breeding and genomic communities in general. The primary reason for choosing the genetic mapping population reported here was to reveal a large number of mapped markers which will be useful in studying the consequences of gene flow from cultivated lettuce into the wild on contamination of the wild germplasm and associated fitness effects, as a model for GM crop release. Like several other crop species cultivated lettuce co-exists with wild sibling species, with which it can interbreed. Further studies are currently underway using this map to screen for selection of crop-specific genomic segments in semi-natural experimental conditions (D.A.P. Hooftman et al., unpublished data). The good distribution of

SSAP markers on the map described here will aid this analysis. In other crops it has been found that SSAP markers have higher polymorphism rates than AFLP markers and therefore perform better in the analysis of genetic diversity (Ellis et al. 1998; Queen et al. 2004). Inclusion of SSAP markers has therefore increased the potential usefulness of this lettuce map for biodiversity studies in lettuce.

**Acknowledgements** This work was supported by Grant 01657 (ANGEL) from the European Commission under the Frameworks VI Programme. We are very grateful to Richard Michelmore, Maria Truco and Alex Kozic who generously provided us with lettuce EST data filtered for transposon sequences prior to publication, together with much helpful advice on linkage mapping in lettuce. We are also thankful for many interesting and fruitful discussions from the other members of the ANGEL partnership—Rikke Bagger Jørgensen, François Felber, Jeroen Rouppe Van der Voort, Gerard Ostermeier and Massimo Vischi.

## References

- Bernatsky R, Tanksley SD (1989) Restriction fragments as molecular markers for germplasm evaluation and utilisation. In: Brown AHD, et al. (eds) *The use of plant genetic resources*. Cambridge University Press, Cambridge, pp 353–362
- Calenge F, Van der Linden CG, Van de Weg E, Schouten HJ, Van Arkel G, Denancé C, Durel CE (2005) Resistance gene analogues identified through the NBS-profiling method map close to major genes and QTL for disease resistance in apple. *Theor Appl Genet* 110:660–668
- Casa AM, Brouwer C, Nagel A, Wang L, Zhang Q, Kresovich S, Wessler SR (2000) Inaugural article: the MITE family heart-breaker (Hbr): molecular markers in maize. *Proc Natl Acad Sci USA* 97:10083–10089
- Castiglioni P, Ajmone-Marsan P, Wijk R (1999) AFLP markers in a molecular linkage map of maize: codominant scoring and linkage group distribution. *Theor Appl Genet* 99:425–431
- Ellis TH, Poyser SJ, Knox MR, Vershinin AV, Ambrose MJ (1998) *Ty1-copia* class retrotransposon insertion site polymorphism for linkage and diversity analysis in pea. *Mol Gen Genet* 260:9–19
- Flavell AJ, Knox MR, Pearce SR, Ellis TH (1998) Retrotransposon-based insertion polymorphisms (RBIP) for high throughput marker analysis. *Plant J* 16:643–650
- Gray AJ (2004) Ecology and government politics: the GM crop debate. *J Appl Ecol* 41:1–10
- Gribbon BM, Pearce SR, Kalendar R, Schulman AH, Paulin L, Jack P, Kumar A, Flavell AJ (1999) Phylogeny and transpositional activity of *Ty1-copia* group retrotransposons in cereal genomes. *Mol Gen Genet* 261:883–891
- Hails RS (2000) Genetically modified plants—the debate continues. *Trends Ecol Evol* 15:14–18
- Haanstra JPW, Wye C, Verbakel H, Meijer-Dekens F, Van den Berg P, Odinet P, Van Heusden AW, Tanksley S, Lindhout P, Peleman P (1999) An integrated high-density RFLP-AFLP map of tomato based on two *Lycopersicon esculentum* × *L. pennellii* F<sub>2</sub> populations. *Theor Appl Genet* 99:254–271
- Hooftman DAP, Oostermeijer JGB, Jacobs M, Den Nijs HCM (2005) Demographic vital rates determine the performance advantage of crop-wild hybrids in Lettuce. *J Appl Ecol* (in press)
- Jeuken M, van Wijk R, Peleman P, Lindhout P (2001) An integrated interspecific AFLP map of lettuce (*Lactuca*) based on two *L. sativa* × *L. saligna* F<sub>2</sub> populations. *Theor Appl Genet* 103:638–647
- Kesseli RV, Paran I, Michelmore RW (1994) Analysis of a detailed genetic linkage map of *Lactuca sativa* (Lettuce) constructed from RFLP and RAPD markers. *Genetics* 136:1435–1446

- Klein PE, Klein RR, Cartinhour SW, Ulanich PE, Dong J, Obert JA, Morishige DT, Schlueter SD, Childs KL, Ale M, Mullet JE (2000) A high-throughput AFLP-based method for constructing integrated genetic and physical maps: progress toward a sorghum genome map. *Genome Res* 10:789–807
- Knapp SJ (1998) Marker-assisted selection as a strategy for increasing the probability of selecting superior genotypes. *Crop Sci* 38:1164–1174
- Korswagen HC, Durbin RM, Smits MT, Plasterk RHA (1996) Transposon Tc1-derived, sequence-tagged sites in *Caenorhabditis elegans* as markers for gene mapping. *Proc Natl Acad Sci USA* 93:14680–14685
- Kosambi DD (1943) The estimation of map units from recombination values. *Ann Eugen* 12:172–175
- Lande R, Thompson R (1990) Efficiency of markers—assisted selection in the improvement of quantitative traits. *Genetics* 124:743–756
- Leigh F, Kalendar R, Lea V, Lee D, Donini P, Schulman AH (2003) Comparison of the utility of barley retrotransposon families for genetic analysis by molecular marker techniques. *Mol Genet Genomics* 269:464–474
- Mantovani P, van der Linden G, Maccaferri M, Sanguineti MC, Tuberosa R (2004) Molecular characterization of durum wheat germplasm with NBS markers as compared to AFLPs and SSRs. In: Vollmann J, Grausgruber H, Ruckebauer P (eds) Proceedings of 17th EUCARPIA General congress: genetic variation for plant breeding. University of Natural Resources and Applied Life Sciences, Vienna, pp 146
- Mei M, Syed NH, Gao W, Thaxton P, Stelly D, Chen ZJ (2004) Genetic mapping and QTL analysis of fiber-related traits in cotton (*Gossypium*). *Theor Appl Genet* 108:280–291
- Meyers BC, Chin DB, Shen KA, Sivaramakrishnan S, Lavelle DO, Zhang Z, Michelmore RW (1998) The major resistance gene cluster in lettuce is highly duplicated and spans several megabases. *Plant Cell* 10:1817–1832
- Nagata RT (1992) Clip-and-wash method of emasculation for lettuce. *Hortscience* 27:907–908
- Pearce SR, Stuart-Rogers C, Knox MR, Kumar A, Ellis TH, Flavell AJ (1999) Rapid isolation of plant Ty1-copia group retrotransposon LTR sequences for molecular marker studies. *Plant J* 19:711–717
- Pearce SR, Knox M, Ellis THN, Flavell AJ, Kumar A (2000) Pea Ty1-copia group retrotransposons: transpositional activity and use as markers to study genetic diversity in *Pisum*. *Mol Genet Genet* 263:898–907
- Porceddu A, Albertini E, Barcaccia G, Marconi G, Bertoli FB, Veronesi F (2002) Development of S-SAP markers based on an LTR-like sequence from *Medicago sativa* L. *Mol Genet Genomics* 267:107–114
- Qi X, Stam P, Lindhout P (1998) Use of locus-specific AFLP markers to construct a high-density molecular map in barley. *Theor Appl Genet* 96:376–384
- Queen RA, Gribbon BM, James C, Jack P, Flavell AJ (2004). Retrotransposon-based molecular markers for linkage and genetic diversity analysis in wheat. *Mol Genet Genomics* 271:91–97
- Reeves JC, Chiapparino, E Donini P, Ganai M, Guiard J, Hamrit S, Heckenberger M, Huang X-Q, van Kaauwen M, Kochieva E, Koebner R, Law JR, Lea V, LeClerc V, van der Lee T, Leigh F, van der Linden G, Malysheva L, Melchinger AE, Orford S, Reif JC, Röder M, Schulman A, Vosman B, van der Wiel C, Wolf M, Zhang D (2004) Changes over time in the genetic diversity of four major European crops from the Gediflux Framework 5 project. In: Vollmann J, Grausgruber H, Ruckebauer P (eds) Proceedings of 17th EUCARPIA General congress: genetic variation for plant breeding. University of Natural Resources and Applied Life Sciences, Vienna, pp 3–7
- Ryder EJ (1999) Lettuce, Endive and Chicory. CAB International, Wallingford
- Sebastian RL, Howell EC, King GJ, Marshall DF, Kearsley MJ (2000) An integrated AFLP and RFLP *Brassica oleracea* linkage map from two morphologically distinct doubled-haploid mapping populations. *Theor Appl Genet* 100:75–81
- Saghai-Marouf MA, Soliman KM, Jorgensen RA, Allard RW (1984) Ribosomal DNA spacer length polymorphisms in barley: mendelian inheritance, chromosomal location, and population dynamics. *Proc Natl Acad Sci USA* 81:8014–8018
- Schulman AH, Flavell AJ, Ellis THN (2004) The application of LTR retrotransposons as genetic markers in plants. In: Miller W, Capy P (eds) Mobile genetic elements: protocols and genomic applications. Humana Press Inc, Totowa,
- Snow AA, Andow DA, Gepts P, Hallerman EM, Power A, Tiedje JM, Wolfenbarger LL (2005) Genetically engineered organisms and the environment: current status and recommendations. *Ecol Appl* 15:377–404
- Stam P, Van Ooijen JW (1995) JoinMap version 2.0: software for the calculation of genetic linkage maps. PRI-DLO, Wageningen
- Syed NH, Sureshsundar S, Wilkinson MJ, Bhau BS, Cavalcanti JJV, Flavell AJ (2005) Ty1-copia retrotransposon-based SSAP marker development in Cashew (*Anacardium occidentale* L.). *Theor Appl Genet* 110:1195–1202
- Van de Wiel C, Van der Linden G, Hans den Nijs, Flavell AJ, Syed N, Jorgensen R, Felber F, Scotti I, Van der Voort JR, Peleman J (2003) An EU project on gene flow analysis between crop and wild forms of lettuce and chicory. In: van Hintum ThJL, Lebeda A, Pink D, Schut JW (eds) The context of GMO biosafety: Eucarpia leafy vegetables
- Van der Linden CG, Wouters DCAE, Mihalka V, Kochieva EZ, Smulders MJM, Vosman B (2004) Efficient targeting of plant disease resistance loci using NBS profiling. *Theor Appl Genet* 109:384–393
- Virk PS, Pooni HS, Syed NH, Kearsley MJ (1999) Fast and reliable genotype validation using microsatellite markers in *Arabidopsis thaliana*. *Theor Appl Genet* 98:462–464
- Voorrips RE (2002) MapChart, Software for the graphical presentation of linkage maps and QTLs. *J Heredity* 93:77–78
- Vuylsteke M, Mank R, Antonise R, Bastiaans E, Senior ML, Stuber CW, Melchinger AE, Lübberstedt T, Xia XC, Stam P, Zabeau M, Kuiper M (1999) Two high-density AFLP linkage maps of *Zea mays* L.: analysis of distribution of AFLP markers. *Theor Appl Genet* 99:921–935
- Vos P, Hogers R, Bleeker M, Reijans M, van de Lee T, Hornes M, Frijters A, Pot J, Peleman J, Kuiper M, Zabeau M (1995) AFLP: a new technique for DNA fingerprinting. *Nucleic Acids Res* 23:4407–4414
- Waugh R, McLean K, Flavell AJ, Pearce SR, Kumar A, Thomas BB, Powell W (1997) Genetic distribution of *BARE-1*-like retrotransposable elements in the barley genome revealed by sequence-specific amplification polymorphisms (S-SAP). *Mol Gen Genet* 253:687–694
- Young WP, Schupp JM, Keim P (1999) DNA methylation and AFLP marker distribution in the soybean genome. *Theor Appl Genet* 99:785–792
- Yu GX, Wise RP (2000) An anchored AFLP- and retrotransposon-based map of diploid *Avena*. *Genome* 43:736–749