Theor Appl Genet (2004) 109: 384–393
DOI 10.1007/s00122-004-1642-8

ORIGINAL PAPER

C. Gerard van der Linden · Doret C. A. E. Wouters · Virag Mihalka · Elena Z. Kochieva · Marinus J. M. Smulders · Ben Vosman

Efficient targeting of plant disease resistance loci using NBS profiling

Received: 10 December 2003 / Accepted: 23 February 2004 / Published online: 1 April 2004

Abstract The conserved sequences in the nucleotide-binding sites of the nucleotide-binding site-leucine-rich repeat (NBS-LRR) class of disease resistance (R) genes have been used for PCR-based R-gene isolation and subsequent development of molecular markers. Here we present a PCR-based approach (NBS profiling) that efficiently targets R genes and R-gene analogs (RGAs) and, at the same time, produces polymorphic markers in these genes. In NBS profiling, genomic DNA is digested with a restriction enzyme, and an NBS-specific (degenerate) primer is used in a PCR reaction towards an adapter linked to the resulting DNA fragments. The NBS profiling protocol generates a reproducible polymorphic multilocus marker profile on a sequencing gel that is highly enriched for R genes and RGAs. NBS profiling was successfully used in potato with several restriction enzymes, and several primers targeted to different conserved motifs in the NBS. Across primers and enzymes, the NBS profiles contained 50–90% fragments that were significantly similar to known R-gene and RGA sequences. The protocol was similarly successful in other crops (including tomato, barley, and lettuce) without modifications. NBS profiling can thus be used to produce markers tightly linked to R genes and R-gene clusters for genomic mapping and positional cloning and to mine for new alleles and new sources of disease resistance in available germplasm.

Communicated by H.F. Linskens

C. G. van der Linden (✉) · D. C. A. E. Wouters · V. Mihalka · E. Z. Kochieva · M. J. M. Smulders · B. Vosman
Plant Research International B.V., Wageningen University and Research Centre,
Droevendaalsesteeg 1,
6708 PB Wageningen, The Netherlands
e-mail: gerard.vanderlinden@wur.nl
Tel.: +31-317-47993
Fax: +31-317-418094

Present address:
V. Mihalka
SVS Holland BV,
P.O. Box 22, 1600 AA Enkhuizen, The Netherlands

Present address:
E. Z. Kochieva
Vavilov Institute of General Genetics,
3 Gubkina Str.,
117809 Moscow , Russia

Introduction

Plants often use a defense mechanism based on the gene-for-gene principle, which means that the product of a resistance (R) gene recognizes a specific avirulence gene product specified by the pathogen (Flor 1971; Keen 1990). A number of these receptor-like R genes have been cloned and characterized. Based on these genes, several classes of R genes can be distinguished. Most R genes identified up till now are members of the cytoplasmic nucleotide-binding site-leucine-rich repeat (NBS-LRR)-containing R-gene family. The NBS region is thought to be important for ATP binding and overall functionality of the R-gene product (Walker et al. 1982; Saraste et al.1990). The LRRs may be the main determinant in recognition of the avirulence gene product (Kobe and Deisenhofer 1995; Jones and Jones 1996; Jia et al. 2000). The NBS-LRR gene family is divided in two subclasses. One class has an upstream conserved domain that resembles the Toll-interleukin receptor (TIR) domain. This class includes the *N* gene from tobacco, *M* and *L6* from flax, and *RPP5* from *Arabidopsis thaliana* (Whitham et al. 1994; Lawrence et al. 1995; Anderson et al. 1997; Parker et al.1997). Members of the other group (including *RPM1* from *Arabidopsis*, *Dm3* from lettuce, *Rx1* and *Gpa2* from potato, and *Prf* and *Mi* from tomato) often contain the consensus for a coiled-coil domain structure at a similar position (Salmeron et al. 1996; Bent et al. 1997; Botella et al. 1998; Meyers et al. 1998; Milligan et al. 1998; van der Vossen et al. 2000). NBS-containing R genes are numerous in plant genomes—163 in *Arabidopsis* and over 600 in rice (TAGI 2000; Goff et al. 2002)—and are often organized in clusters. Within these clusters, repeats of similar genes (paralogues) as well as several different

genes can be recognized (Michelmore and Meyers 1998; Young 2000).

The NBS regions of characterized R genes and of R-gene analogs (RGAs) share several common motifs that are highly conserved. These include the P loop (phosphate-binding domain), the kinase-2 motif, and the GLPL motif (Saraste et al. 1990; Traut 1994; Meyers et al. 1999). These motifs have been widely used to clone NBS regions of RGAs from a variety of species, primarily by amplifying sequences between two motifs (Aarts et al. 1998; Collins et al. 1998; Leister et al. 1998; Shen et al. 1998; Mago et al. 1999; Deng et al. 2000; Noir et al. 2001; Vicente and King 2001). A number of these sequences were mapped and shown to be genetically linked to known R genes, indicating that genomic regions likely to specify resistance, or even candidate R genes, can be identified through cloning and mapping of new RGA sequences (Meyers et al. 1999; Timmerman-Vaughan et al. 2000). In most cases, mapping of these amplified RGA fragments required cloning of the fragments, finding genetic polymorphisms, and turning these polymorphisms into a marker that could be used in segregating populations.

Here we describe an approach (termed "NBS profiling") that generates a large collection of R-gene and RGA fragments and at the same time samples genetic variation in these genes, thus providing molecular markers that are tightly linked to R genes and RGAs. NBS profiling is based on amplification from conserved NBS motifs towards restriction enzyme sites. Our results demonstrate that NBS profiling generates multiple fragments, the majority of which is sequence-related to NBS-containing R genes and RGAs. We show here that this approach can be applied to a wide range of crops without modifications.

Materials and methods

Plant material

Potato (*Solanum tuberosum* L.) and barley (*Hordeum vulgare* L.) varieties were obtained from the Scottish Crop Research Institute, Dundee, Scotland. Tomato (*Lycopersicon esculentum* Mill.) and lettuce (*Lactuca sativa* L.) varieties were from the Centre for Genetic Resources, The Netherlands. Seeds of tomato and barley were allowed to germinate, and young leaves were harvested and lyophilized. Potato tubers were cut in half, allowed to germinate, and leaves of young plants were sampled and lyophilized. For lettuce, young leaves were collected from individual young plants.

Methods

Leaf material from seedlings was lyophilized. DNA was isolated essentially as described by Fulton et al. (1995). Four hundred nanograms of DNA was digested with a restriction enzyme in the appropriate reaction buffer for a total of 4 h, with fresh enzyme added after 2 h. The reaction was terminated by heat inactivation.

An adapter was ligated to the ends of the restriction fragments. This adapter was based on the one described in Fischer et al. (1995), but the 3′ end of the short strand was blocked for extension with *Taq* DNA polymerase by the presence of an amino group. The 5′ end was phosphorylated to facilitate ligation to blunt-end fragments. For *Mse*I ligations, the short strand of the adapter was extended to match the *Mse*1 restriction fragments. Adapter ligation was performed using high-concentrate ligase (5 U/μl) for 16 h at 20°C. The reaction was terminated by heat inactivation.

Amplification of NBS-specific fragments involved a two-step PCR procedure. The first step was a linear (asymmetric) PCR in a PTC-200 thermocycler (MJ Research) with a limited amount of the NBS-specific primer (1.5 mM), 10 μM dNTPs, 0.4 U HotStarTaq (Qiagen, Germany), and 2.5 μl HotStarTaq PCR buffer in a reaction volume of 25 μl. The program consisted of 30 cycles of 30 s at 95°C, 1 min 40 s at 55–60°C annealing, and 2 min at 72°C. Annealing temperature was 55°C for NBS5 and NBS7 primers and 60°C for NBS2. The asymmetric PCR was followed by an exponential PCR with NBS primer and an adapter primer by adding to the linear PCR product: 15 pmol of each primer, 200 μM dNTPs, 0.4 U HotStarTaq, and 2.5 μl HotStarTaq PCR buffer in an end volume of 50 μl. The cycling program and thermocycler were similar to that of the linear PCR.

Finally, the PCR products were labeled by primer extension using the [γ -^{33}P]ATP-end-labeled NBS-specific primer in a thermocycler for 10 cycles with HotStarTaq at conditions similar to the NBS-adapter primer PCR. The labeled PCR products were separated on a 6% polyacrylamide gel, and the individual fragments visualized by autoradiography.

Adapter and adapter primers

- Adapter long arm: 5′-ACTCGATTCTCAACCCGAAAGTATAGATCCCA-3′
- Adapter short arm: 5′-TGGGATCTATACTT-3′ (with 3′ amino group)
- Adapter primer: 5′-ACTCGATTCTCAACCCGAAAG-3′
- NBS-selective primers (location and orientation in NBS, see Fig. 1):

NBS2 5′-GTWGTYTTICCYRAICCISSCAT-3′
NBS5 5′-YYTKRTHGTMITKGATGATGTITGG-3′
NBS7 5′-ATTGTTGGRATGGGMGGIMTIGG-3′

Identification of bands

Fragments were excised from the gel using a sharp razor blade, eluted in TE for 5 min at 100°C, and reamplified with the NBS primer and the adapter primer using conditions similar to the exponential PCR protocol. PCR products were checked on agarose gels and purified with Qiaquick PCR purification spin columns (Qiagen). Fragments were directly sequenced using the adapter primer as a sequencing primer with the BigDye Terminator kit and an ABI 3700 automated sequencer from Applied Biosystems (USA). Sequences were identified by comparison with entries in the public protein and nucleotide databases using locally installed BLASTX and BLASTN programs (Altschul et al. 1997).

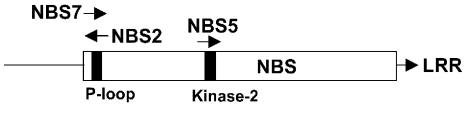

Fig. 1 Schematic representation of the nucleotide-binding site (NBS domain) of disease resistance (R) genes. Positions of the degenerate primers (NBS2, NBS5, and NBS7) used in this study are indicated

Results

Experimental design

The experimental approach described here and termed "NBS-profiling" resembles SSAP (sequence-specific amplification polymorphism) as reported by Waugh et al. (1997) and the R gene-targeted SSAP approach reported by Hayes and Saghai Maroof (2000), but clearly differs at some essential points.

For NBS profiling, genomic DNA was digested with a single restriction enzyme that generated fragments with an average length of 300–400 bp. Next, an adapter was ligated to the ends of the fragments. The adapter had a short and a long arm, leaving the 5′ part of the adapter single stranded. The adapter primer used in the exponential PCR was identical to the single-stranded part of the primer, so adapter primer annealing completely relied on synthesis of a complementary strand. Extension of the 3′ end of the short arm in the subsequent PCR reaction was effectively blocked by an amino linker. The adapter primer can thus only participate in the PCR reaction after extension of the selective NBS primer. Consequently, amplification highly depended on the selectivity and specificity of this NBS primer. To further increase the specificity of the PCR procedure, the actual amplification (exponential PCR) was preceded by asymmetric (linear) PCR with only the NBS primer. In our experience, this asymmetric PCR was essential for obtaining reproducible results. Finally, PCR products were radioactively labeled and separated by polyacrylamide electrophoresis.

Designing PCR primers for targeting RGAs

In the NBS region of R genes, several conserved motifs have been identified (Meyers et al. 1999; Pan et al. 2000), including the P loop, the kinase-2 motif, and the GLPL motif (Fig. 1). For the design of degenerate primers that would recognize a broad spectrum of R genes and RGAs, protein sequences of NBS regions of R genes from a range of species were aligned and conserved amino acid motifs in these sequences identified. These conserved regions (P loop, kinase-2 motif) have been used in a number of studies to isolate NBS regions of R genes and RGAs between two NBS-derived primers. However, the criteria for a primer to be used in our NBS profiling are expected to be different since a single primer, rather than two primers, determines the specificity of the PCR in NBS profiling. Therefore, we aligned the DNA sequences of the conserved motifs of the R genes and based the NBS-profiling primers on this alignment. With several amino acids, a clear codon bias was observed, which allowed minimizing the degree of degeneracy of the primers, while still retaining the ability of the primer to recognize a broad set of RGAs. No clear set of criteria could be formulated to guarantee a successful NBS-profiling primer (success depends on specific sequence of the target region and the putative number of targets), and obtaining a good primer is partly trial and error. We did observe that the success rate was higher when no degeneracy was allowed in the primers within two nucleotides of the 3′ end and the annealing temperature was higher than 50°C. Sequences of primers that produced optimal NBS profiles are given in the Materials and methods section.

Reproducibility, specificity, and polymorphism

The procedure was optimized with material from 12 potato cultivars with a wide genetic background. *Rsa*I was used to digest the DNA of the cultivars. This enzyme produces blunt-ended DNA fragments with an average length of 300–400 bp. NBS profiling was performed with a primer (NBS5) targeted to the kinase-2 motif (Fig. 1), pointing to the 3′ end of the NBS, in combination with the adapter primer. Figure 2 demonstrates that a number of clearly visible bands were present on the gel (typically 50–60 bands), of which at least 15 were polymorphic in the set of potato varieties used. Reproducibility was illustrated by the fact that banding patterns of duplicates (profiles of the same plant material that was split before DNA extraction and processed in separately performed experiments) were identical (Fig. 2).

Bands present in the NBS profile were characterized by sequencing. Seventy six percent of 33 bands collected from several NBS5/*Rsa*I profiles produced a readable sequence by direct sequencing on an ABI 3700 with the adapter primer (a subset of which is indicated in Fig. 2 by arrows). All fragments analyzed (ranging in size from 70 bp to 600 bp) contained an adapter sequence at one end and an NBS primer sequence on the other end, indicating that the procedure efficiently excludes adapter-adapter fragments. The specificity of the two-step PCR procedure is further illustrated by the fact that identical profiling patterns were produced by labeling of the NBS-profiling PCR reaction with either the NBS-selective primer or the adapter primer and subsequent electrophoresis (data not shown).

Sequences of the excised bands were further characterized by sequence similarity searches in the public nucleotide databases using the BLASTN and BLASTX

Table 1 Resistance-gene analog (RGA) frequency in nucleotide-binding site (NBS)-profile bands

	Number of sequenced bands (number of enzymes)	Number of bands with RGA homology	Unidentified	Other genes
NBS2	12 (4)	8	3	1 (retrotransposon)
NBS5	25 (4)	22	2	1 (protein kinase)
NBS7	6 (3)	3	1	Protein kinase; 5S rDNA

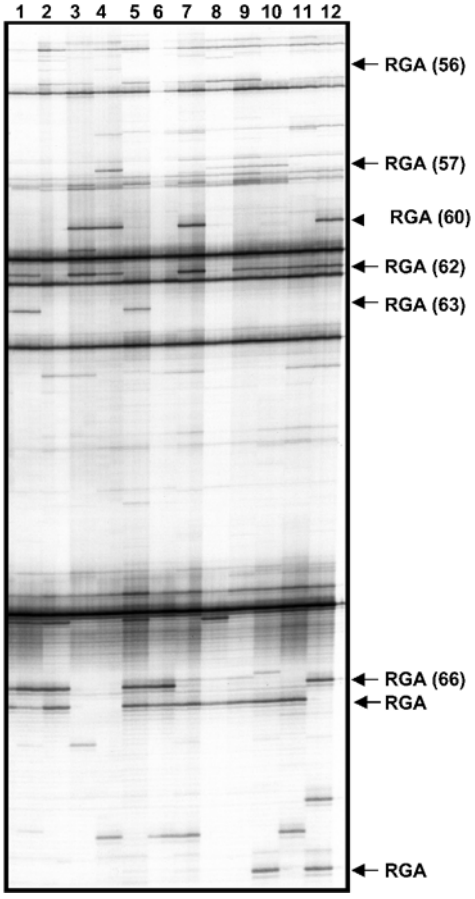

Fig. 2 NBS profile of potato varieties with primer NBS5 and restriction enzyme *Rsa*I. NBS profiling was performed with 12 potato varieties. *Numbers on top* indicate varieties profiled in duplicate (the same DNA sample was used twice in the profiling procedure). A number of bands were excised from the gel, reamplified, and sequenced directly. The sequences from bands indicated by *arrows* (band numbers in *parentheses*) were compared to the nucleotide databases with BLASTX/BLASTN programs (Altschul et al. 1997) (see alignment in Fig. 3). The profile contained fragments with high similarity to known R-gene analogs (RGAs), new RGA sequences, and fragments with sequences that displayed no significant similarity

programs (Altschul et al. 1997). The majority of the sequences (close to 90% for the NBS5 primer, see Table 1) was found to be significantly similar to known NBS sequences of R genes and RGAs of several plant species. These included sequences that were close to identical to known R genes and RGAs, as well as sequences that were significantly similar to other RGAs (35–60% on translated protein sequence) to classify them as such, but clearly were new RGAs, as can be seen in Fig. 3. Band 57 has limited homology to other RGAs, but includes the TTR motif, a characteristic motif for a subset of NBS-LRR genes downstream of the kinase-2 motif, at the expected location.

Expansion of RGA marker set

We performed NBS profiling with a range of frequently cutting restriction enzymes (*Rsa*I, *Mse*I, *Hae*III, *Alu*I) and the NBS5 primer. All of the profiles generated with different restriction enzymes contained reproducible banding patterns (not shown). The majority of the analyzed fragments in each profile were sequence related to NBS-containing R genes or RGAs (based on BLAST analyses), and the degree of polymorphism was comparable between profiles with different restriction enzymes.

We also expanded the NBS-profiling marker set by using other primers. NBS2 and NBS7 are primers designed on the consensus of the P-loop motif, located at the 5′ end of the NBS domain. NBS2 points towards the 5′ end of the NBS; NBS7 points to the 3′ end (Fig. 1). Both primers were tested on potato samples digested with several restriction enzymes and were found to produce polymorphic banding patterns. Bands were characterized by excision from the gel, reamplification and sequencing, and subsequent BLAST similarity searches. In addition, bands were isolated from NBS2 profiles of other crops (barley, tomato). The NBS2-dependent PCR amplified the 5′-flanking region of the NBS of R genes. In contrast to sequences within the NBS (like those of the NBS5 profiles), these sequences are not well conserved between RGAs. Nevertheless, 66% of the fragments generated with

Table 2 NBS-profile bands with high identity to known resistance (R) genes and R-gene cluster members

Species	Band no.	Primer/enzyme	Homologue	Identity-DNA (no. of nucleotides)	Resistance against
Potato	51	NBS3/*Rsa*I	*NL27* (potato)	92% (100)	*Synchytrium endobioticum* (Schilb.) Perc
Potato	162	NBS5/*Hae*III	*Hero* (tomato)	87% (270)	*Globodera pallida* (Stone) Behrens
Tomato	107	NBS5/*Mse*I	*Mi* (tomato)	99% (308)	*Meloidogyne incognita* (Kofoid and White 1919) Chitwood 1949
Tomato	118	NBS2/*Rsa*I	*Mi* (tomato)	93% (270)	*Meloidogyne incognita* (Kofoid and White 1919) Chitwood 1949
Tomato	251	NBS5/*Rsa*I	*Hero* (tomato)	92% (229)	*Globodera pallida* (Stone) Behrens
Tomato	Le 8.6	NBS2/*Rsa*I	*Prf* (tomato)	100% (278)	*Pseudomonas syringae* (van Hall 1902)
Tomato	Le 1.2	NBS2/*Rsa*I	*I2C2* (tomato)	100% (300)	*Fusarium oxysporum* f.sp. *lycopersici*
Barley	89	NBS5/*Rsa*I	*PIC25* (barley)	98% (281)	*Puccinia graminis* Pers.
Lettuce	5.04	NBS5/*Rsa*I	*RCG2* genes (Lettuce)	80–90% (200)	*Bremia lactucae* Regel

NBS2 showed significant similarity to known RGAs and R genes (see Fig. 4 for protein alignment). A small number of bands from the NBS7 primer were sequenced successfully, and half of these were similar to known RGAs.

Transferability to other crops

We have performed NBS profiling on sets of tomato, barley, and lettuce cultivars. In all crops, reproducible polymorphic multilocus patterns were produced using the same or slightly adapted NBS primers and without

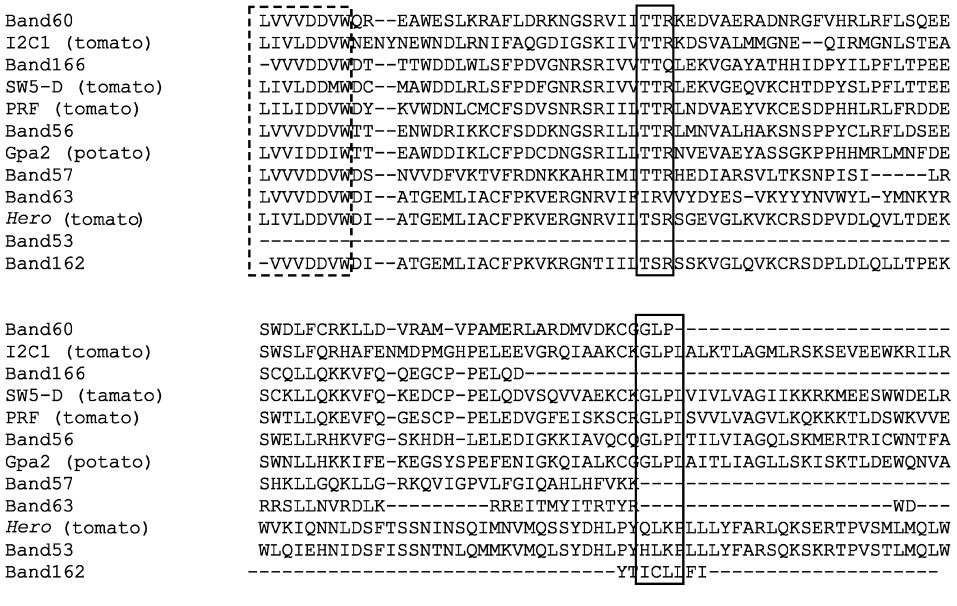

Fig. 3 NBS5 band sequences alignment. Alignment (ClustalW) of translated amino acid sequences of fragments in a potato NBS profiling with primer NBS5 and known R genes and RGAs. Alignment of the tomato *Hero* gene sequence and bands 53 and 162 was performed separately from the other sequences. Band names correspond to the band numbers indicated in Fig. 2. *Lined boxes* indicate amino acid motifs typical for most NBS domains of NBS-LRR R genes (Meyers et al. 1999). The *dashed box* indicates the location of the NBS5 primer

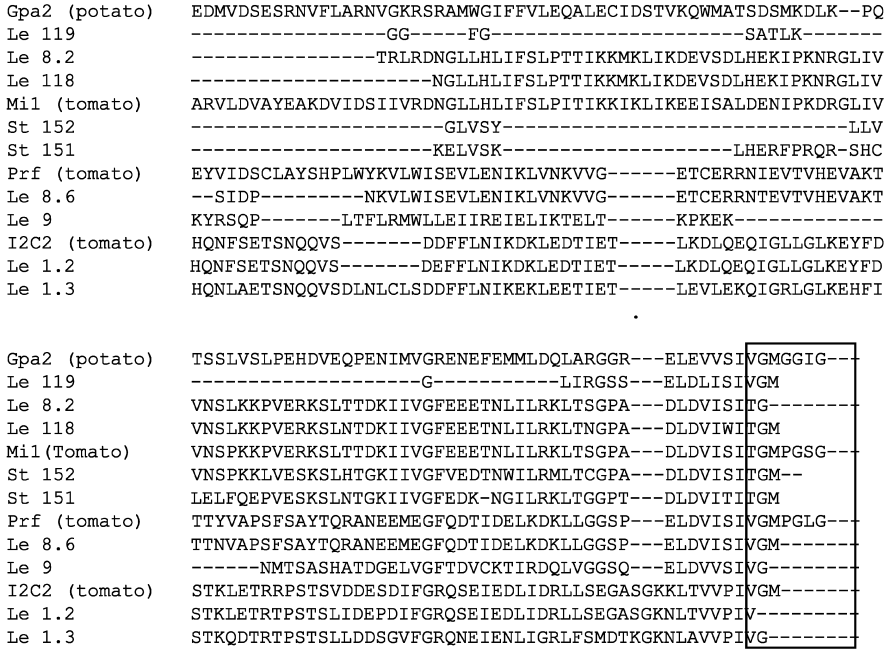

Fig. 4 NBS2 band sequences alignment. Alignment (ClustalW) of translated amino acid sequences of bands excised from NBS2-profiles (tomato and potato) and known R genes and RGAs. The position of the P loop is *boxed*. The NBS2 primer is located in the sequence 3′ flanking the represented sequences, in the P loop

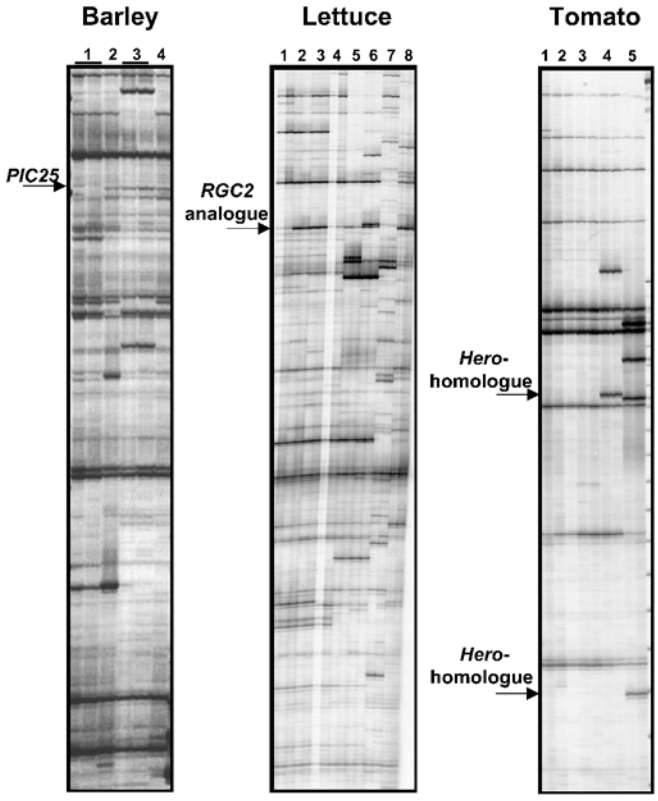

Fig. 5 NBS profiling in different crops. NBS profiles of barley and tomato varieties and lettuce cultivars and wild species (*lanes 1–4*: *Lactuca sativa*, *lanes 5–6*: *L. serriola, lane 7*: *L. saligna*, *lane 8*: *L. virosa*) with primer NBS5 and *Rsa*I (*numbers* indicate single/duplicate samples). *Arrows* indicate bands that were shown to be identical (*PIC25*) or close to identical to already known and mapped R genes and RGAs

modifications to the experimental protocol (Fig. 5). In tomato, we have used the NBS2 primer on a set of tomato cultivars, and analyzed 15 bands from the profiles. Ten bands (67%) were significantly homologous in BLASTX analysis to known R genes and RGAs. These included fragments identical to known R genes (see below; Table 2) as well as new RGAs. Similar results were found with the NBS2 primer. With barley, eight analyzed bands from a profile with another primer from the P-loop motif (directed upstream) yielded four RGA-like sequences. These included a sequence that was 56% identical (protein level, 44 amino acids) to the putative MLA6 protein from rice (accession number AAK52524) and another with 67% identity (protein level, 76 amino acids) to the barley RGA S-9202 (accession number CAD45030). Lettuce profiles with the NBS5 primer included several *RGC2*-like sequences (see also below) as well as *RGC1* and *RGC4* homologues.

Other results from our laboratory indicate that NBS profiling can also be used without modifications in pepper, wheat and apple (data not shown).

NBS profiling markers in known R genes and R-gene clusters

The fragments present in NBS profiles that were (close to) identical to previously characterized R genes, or to RGAs mapped in major R-gene clusters, are listed in Table 2. Several bands from an NBS5/*Rsa*I profile (including band 53 present in the translated protein sequence alignment of Fig. 3) were highly similar (90% or more) to members of the tomato *Hero* gene cluster (Ernst et al. 2002). The *Hero* gene is a broad-spectrum potato cyst nematode R gene that is part of a cluster of at least 14 genes. In tomato, two bands in an NBS5/*Rsa*1 tomato profile also strongly resembled the tomato *Hero* gene (indicated in Fig. 5).

The sequence of a fragment (band 107) from a tomato NBS profiling with NBS5 and *Mse*I as the restriction enzyme was identical to the tomato root knot nematode R gene *Mi* and the *Mi*-copy genes (Milligan et al. 1998; Vos et al. 1998). Sequence identity with *Mi-1*, as well as *Mi-1.1* and *Mi-copy2*, was 304 out of 308 nucleotides. However, the differences were not located at the same positions for each of these genes. Band 107 may therefore originate from another *Mi*-like gene, or be one of the mentioned *Mi* genes, with the differences representing allelic variation between genotypes (none of the genotypes used for isolation of the published *Mi* genes were used for isolation of band 107). The sequence of the tomato band Le 8.6 (see Fig. 4) was 100% identical to the *Prf* gene, which is located in the *Pto* cluster and is essential for resistance to *Pseudomonas syringae* (van Hall 1902) pv. tomato and insecticide sensitivity, together with the *Pto* gene (Salmeron et al. 1996). Band Le 1.2 was identical (100% in 300 bp) to the *I2C2* gene from tomato (see also Fig. 4), a member of the family of *I2* genes that confer resistance to *Fusarium oxysporum* f.sp. *lycopersici* (Ori et al. 1997; Sela-Buurlage et al. 2001).

Barley profiles with NBS5/*Rsa*I contained a polymorphic band (indicated in Fig. 5) with a sequence that was close to identical to the *PIC25* gene (275 bp out of 281 bp identical). The *PIC25* gene maps close the *RPG1* locus for stem rust resistance (Collins et al. 2001).

The sequence of a polymorphic band in a lettuce NBS5/*Rsa*I profile (indicated in Fig. 5) was highly homologous (ca. 90% at both protein and DNA level) to several members of the family of *RGC2* genes of lettuce (Meyers et al. 1998, 1999; Shen et al. 1998). The *RGC2* family members mostly map to a single major resistance cluster in lettuce, which also harbors at least ten R genes for downy mildew (*Dm* genes for *Bremia lactucae* Regel).

Discussion

In the last decade, a number of R genes have been cloned. In most cases, these genes are of the NBS-LRR class of R genes, encoding receptor-like proteins that most likely recognize an avirulence factor and trigger a defense response. Knowledge about the sequences of the large number of R genes present in the plant genome, as well as

the genomic location and organization, will help the understanding of the mechanisms underlying pathogen resistance, which will be of great value for crop improvement strategies related to disease resistance.

In this paper, we describe NBS profiling, a method that efficiently and effectively produces a multilocus marker pattern consisting for the larger part of a diverse collection of R gene and RGA fragments that can easily be isolated from the gel and sequenced. NBS profiling combines the isolation of a wide variety of R genes with monitoring genetic variation in these genes. This opens new possibilities for efficient generation of R gene-associated markers, as well as for evaluation and characterization of germplasm that present new resistance sources.

NBS profiles and markers in resistance loci

The power of NBS profiling was illustrated by the diverse set of RGAs and R genes present as polymorphic markers in the profiles with different primers and enzymes. A single NBS5/*Rsa*I potato profile contained 90% markers with similarity (approximately 50% identity at protein level) to (amongst others) genes from the potato *Gpa2* cluster (*Gpa2*, *RGC1*, *Rx* genes), *RPP13* from *Arabidopsis* (downy mildew resistance), as well as several new RGAs. These RGAs may very well map in close proximity to functional R genes. Numerous RGA sequences have been isolated by others using PCR approaches on conserved NBS motifs (Leister et al. 1996; Aarts et al.1998; Collins et al. 1998; Shen et al. 1998; Mago et al. 1999; Timmerman-Vaughan et al. 2000), and many of these RGAs map close to or within known R-gene clusters.

Within a single potato NBS5/*Rsa*I profile, five bands were found to be highly similar to the tomato *Hero* gene, which confers a high level of resistance to the potato cyst nematode *Globodera pallida* (Stone) Behrens (Ernst et al.2002). It is not unlikely that the genes represented by the NBS-profile bands are members of a potato *Hero*-homologous cluster. The markers display different polymorphic patterns in the varieties tested, indicating that they represent different haplotypes. It will be interesting to see where these markers are located on the potato chromosomal map and whether they co-localize with known clusters and/or quantitative trait loci for *G. pallida* resistance.

A band present in a tomato profile with the NBS2 primer (Le 8.6) was 100% identical to the *Prf* gene sequence published in accession number AF220603 (*P. syringae* pv. tomato-susceptible cultivar VFNT Cherry). The sequenced band was generated with the *Lycopersicon esculentum* Mill. cultivar Moneymaker, which is also susceptible to *Pseudomonas*. Interestingly, this sequence differed at three nucleotides from the sequence of accession number AF220602 [resistant *Pto* locus of *L. pimpinellifolium* (L.) Mill.] and U65391 (mRNA from cultivar Rio Grande with *L. pimpinellifolium Pto* locus introgressed). No stop codons are introduced by the nucleotide substitutions, but two of the three nucleotide changes introduce amino acid substitutions, which may affect correct functioning of the PRF protein.

Methodological considerations

Several factors may contribute to the ability of NBS profiling to target NBS-containing RGA sequences efficiently. Most importantly, the primer needs to be selective for R genes and RGAs, while at the same time recognizing a maximum number of different genes. The success of a primer in the NBS-profiling procedure depends on the number of degeneracies, the length, the nucleotide composition, and the positions of the degeneracies within the primer, as well as the number of putative targets in the genomic DNA tested. The chances of a primer to be successful in NBS profiling appear to be highest when no degeneracies are allowed in the last two positions at the 3′ end and the number of degeneracies is kept to a minimum.

Other steps in the protocol also contribute to the specificity and reliability. The use of a single adapter at both sides with a blocked 3′ end of the short strand was essential for the production of fingerprints with low or no background of adapter-adapter PCR fragments. The asymmetric PCR with the selective primer that precedes the adapter-to-NBS PCR was shown to enhance reproducibility. Furthermore, the use of a *Taq* polymerase with a hot start appeared to be essential for obtaining high-quality fingerprints (not shown).

As specificity of NBS profiling relies on a single degenerate NBS-specific primer that is designed on the basis of an alignment of known R-gene sequences, it is conceivable that the primer selectivity is biased towards already known R genes. For the profiles with the NBS primers used in this study, more than half of the sequenced bands that were classified as RGAs were structurally similar to other RGAs (containing other conserved motifs, overall similarity at protein level close to 50%), but not highly similar to known R genes. This demonstrates that although identified R genes were targeted (see Table 2), a significant proportion of new RGAs is also present in the profiles. A bias towards known R genes may still be present, but NBS profiling as presented in this paper produces a large fraction of new RGAs as well.

Using different primers

Primers targeted to the same motif but at slightly different positions and with slightly different nucleotide compositions can produce completely different NBS-profiling fingerprints, indicating that the genetic variation detected by NBS profiling can be increased by adapting primer sequences to target new RGAs. The NBS5 primer recognizes the kinase-2 domain and is directed to the 3′ part of the RGAs. The 3′ end of the primer is based on the highly conserved amino acid sequence DD(I/V)W. Conservation of the tryptophan at this location is tightly linked

to the class of non-TIR R genes (Meyers et al. 1999; Pan et al. 2000). Targeting the TIR-containing R-gene family would require a primer that is also targeted at the kinase-2 domain, but with a adapted 3′ end to match the DD(I/V)D conserved sequence of this subclass of R genes. In the *Arabidopsis* genome, 30% of 161 putative NBS-containing R genes are of the non-TIR class (TAGI 2000). Interestingly, the R genes and RGAs identified in monocotyledonous species up until now are exclusively of the non-TIR class (Pan et al. 2000). The NBS5 primer should be able to target NBS-containing genes in monocots as well as dicots, which is confirmed by the fact that in barley, we have identified in profiles with NBS5 a number of RGAs. A DD(I/V)D-targeted primer would potentially recognize more R genes and RGAs in dicotyledons, but may be useless in monocots.

Another possibility of expanding the RGA-targeted marker set would be to sample genetic variation in the LRR of NBS-LRR genes. A high mutation rate in the LRR contributes to a genetic source of a large set of pathogen specificities (Michelmore and Meyers 1998; Sicard et al. 1999). This may be possible by targeting the (not-so-well conserved) motifs downstream of the kinase-2 domain—for instance, the GLPL motif. Alternatively, the NBS5 primer may be used in an NBS profiling with restriction enzymes that cut less frequent, generating longer fragments that run into the LRR region.

With sequence information accumulating in a number of crops, it should be possible to further optimize primer sequences to efficiently target RGAs in these crops and even target specific clusters or subsets of R genes and RGAs. The primers presented in this paper appear to be widely applicable, with good results (polymorphic profiles with sufficient number of bands and a large fraction of bands related to RGA sequences) in a number of crops. In addition to the Solanaceae species (potato, pepper and tomato), NBS profiles with these primers were produced for cultivars and wild relatives of amongst others barley, lettuce, apple, wheat, and rice.

Other RGA-targeting marker approaches

Several other approaches have been published that use conserved regions in R genes and RGAs to assess biodiversity at disease resistance loci. Chen et al. (1998) used a PCR approach with degenerate primer pairs to amplify part of the NBS of a number of RGAs as reported by Leister et al. (1996) and others, and length polymorphisms of the PCR product were detected with the high-resolution electrophoresis RGA polymorphism technique. NBS profiling differs from this approach by using a single degenerate RGA primer in combination with an adapter ligated to a restriction-enzyme site. This enables the detection of length polymorphisms as well as polymorphisms within the recognition site of the restriction enzyme. The possibility to screen the targeted RGAs and R genes with a number of restriction enzymes increases the chances of finding genetic variation that is closely linked to the resistance trait of interest.

The approach used for NBS profiling has similarities to the modified AFLP approach of Hayes et al. (2000), which is based on the SSAP protocol introduced by Waugh et al. (1997) for targeting the *BARE-1* transposon in barley, and to ligation-mediated PCR (LM-PCR) (Hornstra and Yang 1993). In the modified AFLP approach, digestion of the DNA, adapter ligation, and the pre-amplification steps are according to the AFLP protocol. Subsequently, the PCR reaction with the (labeled) *Eco*R1 primer and a degenerate NBS-specific primer generates the PCR fragments of the fingerprint. However, the frequency of RGAs in fingerprints based on the modified AFLP procedure with degenerate NBS-selective primers was at best 20–25% (Hayes et al. 2000). In contrast, NBS profiles of potato with the NBS5 primer contained 88% sequenced fragments that could be positively identified as NBS-containing RGAs. Across primers, enzymes, and species, the NBS-profiles contained 40–90% RGAs.

LM-PCR was introduced as a fingerprinting technique by Hornstra and Yang (1993) and adapted by Trognitz et al. (2002) for targeting defense-related genes. The LM-PCR strategy in the latter study utilized primers designed to target defense-related genes and produced bands (13%) that were associated with QTLs for resistance. Trognitz et al. did not use the LM-PCR technique in combination with primers targeted to the NBS-LRR genes. LM-PCR significantly differs from NBS profiling in the use of six basepair-recognizing restriction enzymes rather that frequently cutting enzymes and the use of less degenerate gene-specific primers. This results in footprints that consist of a single or just a few loci per assay, in contrast to the multi-locus profile of NBS profiling.

Prospects of NBS profiling

The results presented in this paper clearly demonstrate that NBS profiling is a powerful technique that produces markers in R genes and RGAs with relative ease. It can be used across species with little or no modifications to primers or protocol. NBS profiling can therefore be an important new tool in crop improvement strategies, germplasm characterization, and biodiversity studies.

NBS profiling can be a gateway to reliable markers for disease resistance. In segregating crosses, NBS profiling can be used to tag disease R genes. Starting with a bulked segregant analysis, primer-enzyme combinations producing polymorphic bands can be quickly identified. Markers that cosegregate with resistance can be further characterized and possibly converted into a high-throughput, codominantly scorable marker. Preliminary results indicate that polymorphic loci can be easily mapped on the genome using a segregating population in several crops. By carefully choosing the primer sequences, it is possible to target subsets of R genes and specific clusters, allowing an even more focused search for markers closely linked to a disease resistance locus. In addition, new members of

existing clusters as well as new putative R-gene clusters can thus be identified. It is anticipated that with the availability of a BAC library for the genome under investigation, NBS profiling or NBS profiling-derived markers may be used to identify a specific BAC clone that can then be further characterized.

Molecular markers are used to characterize collections. NBS profiling can be used to identify genetic diversity in resistance loci. Collections can thus be characterized for this type of important characters. This will facilitate a more efficient use of the collections. In addition, wild relatives of agronomically important crops can be specifically mined for new resistance sources.

At least part of the markers in an NBS profile will most likely be under selection. This will have implications for the way the profiling marker data can be used in ecological and phylogenetic biodiversity studies as discussed by van Tienderen et al. (2002). Comparative studies of NBS profiling and random marker systems like AFLP on the same set of genotypes may shed light on the role of disease R genes in speciation.

Targeting other gene families: motif-directed profiling

NBS profiling enables the amplification and generation of molecular markers in or near R genes and RGAs. The approach can easily be extended to gene families. This so-called motif-directed profiling may be carried out on any set of targets for amplification in the genome with sufficient conservation to allow selective binding of a (degenerate) primer. Targets may include interesting gene families in plants as well as other organisms (including humans), such as protein kinases, cytochrome P450 genes, and MADS-box genes, as well as other conserved DNA motifs like promoter elements involved in regulation of gene transcription.

Acknowledgements The authors wish to thank Dr. Edwin van der Vossen for critically reading the manuscript. This study has been carried out with financial support from the Ministry of Agriculture, Nature Management and Fisheries of The Netherlands (DLO program 283) and from the Commission of the European Communities, contract BIO4-98-033. It does not necessarily reflect its views and in no way anticipates the Commission's future policy in this area.

References

Aarts MGM, Te Lintel Hekkert B, Holub EB, Beynon JL, Stiekema WJ, Pereira A (1998) Identification of R-gene homologous DNA fragments genetically linked to disease resistance loci in *Arabidopsis thaliana*. Mol Plant Microbe Interact 11:251–258

Altschul SF, Madden TL, Schäffer AA, Zhang J, Zhang Z, Miller W, Lipman DJ (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res 25:3389–3402

Anderson PA, Lawrence GJ, Morrish BC, Ayliffe MA, Finnegan EJ, Ellis JG (1997) Inactivation of the flax rust resistance gene *M* associated with loss of a repeated unit within the leucine-rich repeat coding region. Plant Cell 9:641–651

Bent AF, Kunkel B, Dahlbeck D, Brown KL, Schmidt R, Giraudat J, Leung J, Staskawicz BJ (1997) *RPS2* of *Arabidopsis thaliana*: a leucine-rich repeat class of plant disease resistance genes. Science 265:1856–1860

Botella M A, Parker J E, Frost LN, Bittner-Eddy PD, Beynon JL, Daniels MJ, Holub EB, Jones JDG (1998) Three genes of the *Arabidopsis RPP1* complex resistance locus recognize distinct *Peronospora parasitica* avirulence determinants. Plant Cell 10:1847–1860

Chen XM, Line RF, Leung H (1998) Genome scanning for resistance-gene analogs in rice, barley, and wheat by high-resolution electrophoresis. Theor Appl Genet 97:345–355

Collins NC, Webb CA, Seah S, Ellis JG, Hulbert SH, Pryor A (1998) The isolation and mapping of disease resistance gene analogs in maize. Mol Plant Microbe Interact 11:968–978

Collins NC, Park R, Spielmeyer W, Ellis J, Pryor T (2001) Resistance gene analogs in barley and their relationships to rust resistance genes. Genome 44:375–381

Deng Z, Huang S, Ling P, Chen C, Yu C, Weber CA, Moore GA, Gmitter FG Jr (2000) Cloning and characterization of NBS-LRR class resistance-gene candidate sequences in citrus. Theor Appl Genet 101:814–822

Ernst K, Kumar A, Kriseleit D, Kloos DU, Phillips MS, Ganal MW (2002) The broad-spectrum potato cyst nematode resistance gene (*Hero*) from tomato is the only member of a large gene family of NBS-LRR genes with an unusual amino acid repeat in the LRR region. Plant J 31:127–136

Fischer A, Saedler H, Theissen G (1995). Restriction fragment length polymorphism-coupled domain-directed differential display: a highly efficient technique for expression analysis of multigene families. Proc Natl Acad Sci USA 92:5331–5335

Flor HH (1971) Current status of the gene-for-gene concept. Annu Rev Phytopathol 9:275–276

Fulton TM, Chunwongse J, Tanksley SD (1995) Microprep protocol for extraction of DNA from tomato and other herbaceous plants. Plant Mol Biol Rep 13:207–209

Goff SA, Ricke D, Lan TH, Presting G, Wang R, Dunn M, Glazebrook J, Sessions A, Oeller P, Varma H, Hadley D, Hutchison D, Martin C, Katagiri F, Lange BM, Moughamer T, Xia Y, Budworth P, Zhong J, Miguel T, Paszkowski U, Zhang S, Colbert M, Sun WL, Chen L, Cooper B, Park S, Wood TC, Mao L, Quail P, Wing R, Dean R, Yu Y, Zharkikh A, Shen R, Sahasrabudhe S, Thomas A, Cannings R, Gutin A, Pruss D, Reid J, Tavtigian S, Mitchell J, Eldredge G, Scholl T, Miller RM, Bhatnagar S, Adey N, Rubano T, Tusneem N, Robinson R, Feldhaus J, Macalma T, Oliphant A, Briggs S (2002) A draft sequence of the rice genome (*Oryza sativa* L. ssp. *japonica*). Science 296:92–100

Hayes AJ, Saghai Maroof MA (2000) Targeted resistance gene mapping in soy bean using modified AFLPs. Theor Appl Genet 100:1279–1283

Hornstra IK, Yang TP (1993) In vivo footprinting and genomic sequencing by ligation-mediated PCR. Anal Biochem 213:179–193

Jia Y, McAdams SA, Bryan GT, Hershey HP, Valent B (2000) Direct interaction of resistance gene and avirulence gene products confers rice blast resistance. EMBO J 19:4004–4014

Jones DA, Jones JDG (1997) The role of leucine-rich repeat proteins in plant defenses. Adv Bot Res 24:89–167

Keen NT (1990) Gene-for-gene complementarity in plant-pathogen interactions Annu Rev Genet 24:447–463

Kobe B, Deisenhofer J (1994) The leucine-rich repeat: a versatile binding motif. Trends Biochem Sci 19:415–421

Lawrence GJ, Finnegan EJ, Ayliffe MA, Ellis JG (1995) The *L6* gene for flax rust resistance is related to the *Arabidopsis* bacterial resistance gene *RPS2* and the tobacco viral resistance gene *N*. Plant Cell 7:1195–206

Leister D, Ballvora A, Salamini F, Gebhardt C (1996) A PCR-based approach for isolating pathogen resistance genes from potato with potential for wide application in plants. Nat Genet 14:421–429

Leister D, Kurth J, Laurie DA, Yano M, Sasaki T, Devos K, Graner A, Schulze-Lefert P (1998) Rapid reorganization of resistance gene homologues in cereal genomes Proc Natl Acad Sci USA 95:370–375

Mago R, Nair S, Mohan M (1999). Resistance gene analogues from rice: cloning, sequencing and mapping. Theor Appl Genet 99:50–57

Meyers BC, Chin DB, Shen KA, Sivaramakrishnan S, Lavelle DO, Zhang Z, Mitchelmore RW (1998) The major resistance gene cluster in lettuce is highly duplicated and spans several megabases. Plant Cell 10:1817–1832

Meyers BC, Dickerman AW, Michelmore RW, Sivaramakrishnan S, Sobral B, Young ND (1999) Plant disease resistance genes encode members of an ancient and diverse protein family within the nucleotide-binding superfamily. Plant J 20:317–332

Michelmore RW, Meyers BC (1998) Clusters of resistance genes in plants evolve by divergent selection and a birth-and-death process. Genome Res 8:1113–1130

Milligan SB, Bodeau J, Yaghoobi J, Kaloshian I, Zabel P, Williamson VM (1998) The root knot nematode resistance gene *Mi* from tomato is a member of the leucine zipper, nucleotide-binding, leucine-rich repeat family of plant genes. Plant Cell 10:1307–1319

Noir S, Combes M-C, Anthony F, Lashermes P (2001) Origin, diversity and evolution of NBS-type disease-resistance gene homologues in coffee trees (*Coffea* L.). Mol Genet Genom 265:654–662

Ori N, Eshed Y, Paran I, Presting G, Aviv D, Tanksley S, Zamir D, Fluhr R (1997) The *I2C* family from the wilt disease resistance locus *I2* belongs to the nucleotide-binding, leucine-rich repeat superfamily of plant resistance genes. Plant Cell 9:521–532

Pan Q, Wendel J, Fluhr R (2000) Divergent evolution of plant NBS-LRR resistance gene homologues in dicot and cereal genomes. J Mol Evol 50:203–213

Parker JE, Coleman MJ, Szabo V, Frost LN, Schmidt R, van der Biezen EA, Moores T, Dean C, Daniels MJ, Jones JDG (1997) The *Arabidopsis* downy mildew resistance gene *RPP5* shares similarity to the Toll and interleukin-1 receptors with *N* and *L6*. Plant Cell 9:879–894.

Salmeron J, Oldroyd G, Rommens C, Scofield S, Kim H, Lavelle D, Dahlbeck D, Staskawicz B (1996) Tomato *Prf* is a member of the leucine-rich repeat class of plant disease resistance genes and lies embedded within the *Pto* kinase gene cluster. Cell 86:123–133

Saraste M, Sibbald PR, Wittinghofer A (1990). The P-loop: a common motif in ATP- and GTP-binding proteins. Trends Biochem Sci 15:430–434

Sela-Buurlage MB, Budai-Hadrian O, Pan Q, Carmel-Goren L, Vunsch R, Zamir D, Fluhr R (2001) Genome-wide dissection of fusarium resistance in tomato reveals multiple complex loci. Mol Genet Genom 265:1104–1111

Shen KA, Meyers BC, Islam-Faridi MN, Chin DB, Stelly DM, Michelmore RW (1998) Resistance gene candidates identified by PCR with degenerate oligonucleotide primers map to clusters of resistance genes in lettuce. Mol Plant Microbe Interact 11:815–823

Sicard D, Woo S-S, Arroyo-Garcia R, Ochoa O, Nguyen D, Korol A, Nevo E, Mitchelmore R (1999) Molecular diversity at the major cluster of disease resistance genes in cultivated and wild *Lactuca* spp. Theor Appl Genet 99:405–418

The Arabidopsis Genome Initiative (TAGI) (2000) Analysis of the genome sequence of the flowering plant *Arabidopsis thaliana*. Nature 6814:796–815

Tienderen PH van, De Haan AA, Van der Linden CG, Vosman B (2002) Biodiversity assessment using markers for ecologically important traits. Trends Ecol Evol 17:577–582

Timmerman-Vaughan GM, Frew TJ, Weerden NF (2000) Characterization and linkage mapping of R-gene analogous DNA sequences in pea (*Pisum sativum* L). Theor Appl Genet 101:241–247

Traut TW (1994) The functions and conserved motifs of nine types of peptide segments that form different types of nucleotide-binding sites. Eur J Biochem 229: 9–19

Trognitz F, Manosalva M, Gysin R, Nino-Liu D, Simon R, Herrera MR, Trognitz B, Ghislain M, Nelson R (2002) Plant defense genes associated with quantitative resistance to potato late blight in *Solanum phujera* × dihaploid *S. tuberosum* hybrids. Mol Plant Microbe Interact 15:587–597

Vicente JG, King GJ (2001) Characterisation of disease gene-like sequences in *Brassica oleracea* L. Theor Appl Genet 102:555–563

Vos P, Simons G, Jesse T, Wijbrandi J, Heinen L, Hogers R, Frijters A, Groenendijk J, Diergaarde P, Reijans M, Fierens-Onstenk J, de Both M, Peleman J, Liharska T, Hontelez J, Zabeau M (1998) The tomato *Mi-1* gene confers resistance to both root knot nematodes and potato aphids. Nature Biotechnol 16:1365–1369

Vossen EAG van der, Rouppe van der Voort JNAM, Kanyuka K, Bendahmane A, Sandbrink S, Baulcombe DC, Bakker J, Stiekema WJ, Klein-Lankhorst RM (2000) Homologues of a single resistance-gene cluster in potato confer resistance to distinct pathogens: a virus and a nematode. Plant J 23:567–576

Walker JE, Saraste M, Runswick MJ, Gay NJ (1982) Distantly related genes in the α- and β-subunits of ATP synthetase, myosin, kinases and other ATP-requiring enzymes and a common nucleotide-binding fold. EMBO J 1:945–951

Waugh R, McLean K, Flavell AJ, Pearce SR, Kumar A, Thomas BBT, Powell W (1997) Genetic distribution of *Bare-1*-like retrotransposable elements in the barley genome revealed by sequence-specific amplification polymorphisms (SSAP). Mol Gen Genet 253:687–694

Whitham S, Dinesh-Kumar SP, Choi D, Hehl R, Corr C, Baker B (1994). The product of the tobacco mosaic virus resistance gene *N*: Similarity to Toll and the interleukin-1 receptor. Cell 78:1101–1115.

Young ND (2000) The genetic architecture of resistance. Curr Opin Plant Biol 3:285–290