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Resistance gene analogues identified through the NBS-profiling method map close to major genes and QTL for disease resistance in apple

Abstract We used a new method called nucleotide-binding site (NBS) profiling to identify and map resistance gene analogues (RGAs) in apple. This method simultaneously allows the amplification and the mapping of genetic markers anchored in the conserved NBS-encoding domain of plant disease resistance genes. Ninety-four individuals belonging to an F_1 progeny derived from a cross between the apple cultivars 'Discovery' and 'TN10-8' were studied. Two degenerate primers designed from the highly conserved P-loop motif within the NBS domain were used together with adapter primers. Forty-three markers generated with NBS profiling could be mapped in this progeny. After sequencing, 23 markers were identified as RGAs, based on their homologies with known resistance genes or NBS/leucine-rich-repeat-like genes. Markers were mapped on 10 of the 17 linkage groups of the apple genetic map used. Most of these markers were organized in clusters. Twenty-five markers mapped close to major genes or quantitative trait loci for resistance to scab and mildew previously identified in different apple progenies. Several markers could become efficient tools for marker-assisted selection once converted into breeder-friendly markers. This study demonstrates the efficiency of the NBS-profiling method for generating RGA markers for resistance loci in apple.

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Introduction

Several dozens of plant resistance genes have been cloned and sequenced during the last decade (Hammond-Kosack and Parker 2003). Sequence analysis reveals that resistance (R) genes are highly conserved among plant species. Several classes of R genes are defined according to the protein domains they encode (Dangl and Jones 2001; Hammond-Kosack and Parker 2003). The nucleotide-binding site (NBS)/leucine-rich repeat (LRR) class of receptor-like genes is by far the largest class.

Conservation of resistance gene sequences has often been used to isolate resistance gene analogues (RGAs) by PCR, with degenerate primers designed from the highly conserved motifs of these genes. Motifs within the NBS-encoding domain have been by far the most frequently targeted motifs. RGAs have been cloned and mapped in many species, using this method (e.g. Leister et al. 1996; Shen et al. 1998; Collins et al. 1998). These RGAs often map close to major resistance genes or quantitative trait loci (QTL) (e.g. Speulman et al. 1998; Geffroy et al. 2000; Gebhardt and Valkonen 2001), and thus may provide either interesting candidate genes or useful markers for marker-assisted selection (MAS). They also provide information about the organization and evolution of resistance genes and RGAs in plant genomes (Grube et al. 2000; Pan et al. 2000; Bai et al. 2003; Meyers et al. 2003).

Most studies dissociate the isolation (cloning) of RGAs and their genetic mapping in segregating progenies. Most often, the cloned RGAs are mapped by restriction fragment length polymorphism (RFLP), which is often time-consuming. Hayes and Saghai-Maroof (2000), and more recently, Van der Linden et al. (2004), proposed new strategies simultaneously to generate polymorphism and specifically amplify highly conserved motifs. Both methods are based on the simultaneous use of an adapter primer matching a
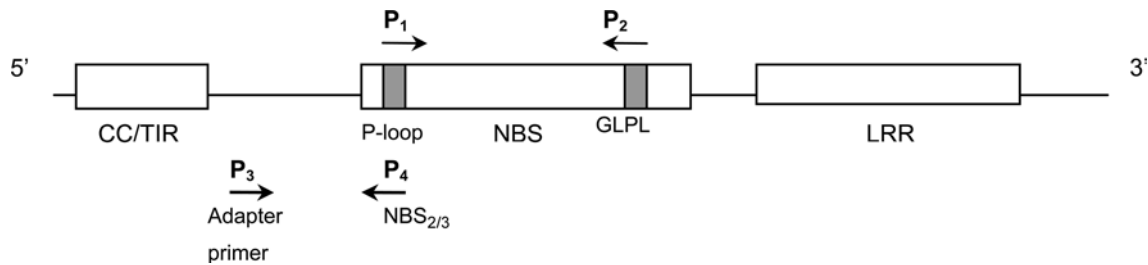
Fig. 1 Schematic representation of the structure of the targeted nucleotide-binding site (*NBS*)/leucine-rich repeat (*LRR*) sequences. Exons are represented by *boxes* and introns by *lines between boxes* (exons and introns are not drawn to scale). *Shaded boxes* indicate highly conserved motifs inside the NBS-encoding region. *Arrows* indicate positions and directions of two distinct sets of primers: P_1

and P_2 represent the primers usually used in other studies to amplify NBS-encoding sequences; they are designed to amplify DNA inside the NBS-encoding region. P_3 and P_4 represent the primers used in the present study; they were designed to amplify DNA outside the NBS-encoding region, towards the 5′ end of the DNA sequence

restriction enzyme site and of a degenerate primer targeting the NBS-encoding region in PCR reactions. One primer is therefore anchored in the NBS-encoding region and genetic variation is sampled in the gene region flanking the primer-binding site. NBS-profiling was shown to be highly effective in generating polymorphic markers with high sequence homology to RGAs in several species (Van der Linden et al. 2004).

This report demonstrates the effective use of the NBS-profiling approach for the mapping of new genetic markers homologous to NBS/LRR genes in apple (*Malus* spp.). In apple, at least 12 major genes have been identified for resistance to scab, caused by the fungal pathogen *Venturia inaequalis* (Williams and Kuc 1969; Lespinasse 1989; Bénaouf and Parisi, 2000; Durel et al. 2000; Hemmat et al. 2002; Patocchi et al. 2003; Tartarini et al. 2003). Most of these have now been mapped. In addition, several QTL for resistance to *V. inaequalis* have been mapped in three apple progenies (Durel et al. 2003; Liebhard et al. 2003b; Calenge et al. 2004). Several major genes for resistance to mildew have also been identified and mapped in apple (Alston 1983; Evans and James 2003). To date, only one resistance gene has been isolated and sequenced in apple: the *HcrVf2* gene, belonging to the *Cf*-like class of resistance genes (Belfanti et al. 2004). Identification and mapping of RGAs in apple might be a way of discovering new sequences related to disease resistance or finding sequences of previously identified genes. A previous study (Baldi et al. 2004) recently reported the isolation of about 50 NBS/LRR homologues in apple. The authors encountered some difficulties in identifying polymorphism; so only 18 markers could finally be mapped. The NBS-profiling method was used in this study to identify and map new RGAs more easily.

The NBS-profiling method was used in an apple F_1 progeny derived from a cross between the cultivar 'Discovery' and the hybrid 'TN10-8'. Our purposes were to: (1) assess the efficiency of the NBS-profiling method to provide polymorphic markers in a segregating progeny; (2) search for genetic co-localizations among the new markers and previously mapped disease resistance loci, in an attempt to characterize these loci; (3) find markers close to major resistance genes for MAS; and

(4) determine the overall genomic organization of some NBS/LRR-like sequences in apple.

Material and methods

Plant materials

A progeny of 149 F_1 individuals was derived from a cross between the apple cultivar 'Discovery' used as the female parent, and the apple hybrid 'TN10-8' used as the male parent (Calenge et al. 2004). 'Discovery' is an English variety derived from a cross between 'Worcester Pearman' and 'Beauty of Bath.' 'TN10-8' is a French hybrid derived from a cross between the scab-tolerant French cultivar 'Reinette Clochard' and a hybrid derived from 'Schmidt's Antonovka P.I. 172632.' The 'Discovery × TN10-8' progeny was sown in 1997 and a genetic linkage map constructed by Calenge et al. (2004). Ninety-four of the 149 individuals in the progeny were randomly chosen for this study.

NBS-profiling of the 'Discovery × TN10-8' progeny

DNA samples of the 'Discovery × TN10-8' progeny and parent lines were isolated using the DNeasy 96 plant kit of Qiagen (Germany). The NBS profiling of the progeny was performed essentially as described previously (Van der Linden et al. 2004). In brief, DNA was digested during 4 h with either the *Rsa*1 or *Mse*1 enzyme, using 400 ng DNA per individual. An adapter was ligated to the restriction fragments. Adapters and adapter sequences were described previously (Van der Linden et al. 2004). Two different degenerate primers, NBS2 (5′-GTWGTYTTICCYRAICCISSCAT-3′) and NBS3 (5′-GTWGTYTTICCYRAICCISSCATICC-3′), were then used for a two-step PCR procedure. These primers were designed from a part of the conserved P-loop motif belonging to the NBS-encoding region of plant disease resistance genes (Van der Linden et al. 2004). Both primers were designed to amplify DNA towards the 5′ end of the targeted genes, outside the NBS domain (Fig. 1). The first PCR was linear, i.e. only the degen-

erate primer was used (either NBS2 or NBS3). The second PCR was exponential, i.e. it was performed with both the degenerate primer and the adapter primer. Both PCRs were performed with an annealing temperature of 60°C on a PTC-200 thermocycler (MJ Research, Waltham, Mass., USA), using the following cycling program: 30 cycles of 30 s 95°C, 1 min 40 s 60°C and 2 min 72°C. The final PCR products were labelled by primer extension, using the $[\gamma^{33}P]$-ATP end-labelled NBS primers for ten cycles at conditions similar to that of the linear PCR. PCR products were separated on 6% polyacrylamide gels for 3 h at 110 W. Gels were then transferred to 3 MM paper covered with plastic wrap and exposed to Kodak Xomat films (New Haven, Conn., USA).

Genetic mapping

Polymorphic bands observed on autoradiographs were scored for their presence/absence in the progeny. They were mapped on an integrated 'female + male' linkage map of the 'Discovery' × 'TN10-8' progeny and on the component, parental linkage maps constructed previously (Calenge et al. 2004), using the JoinMap software, version 2.0 (CPRO-DLO, Wageningen, The Netherlands). Markers were first mapped using the integrated linkage map. Markers that could not be located on this map were mapped on the separated parental maps, following the double pseudo-test cross strategy (Grattapaglia et al. 1994).

Sequence analyses

Polymorphic bands were excised from the polyacrylamide gels, using a sharp razor blade and eluted in 50 μl water for 10 min at 100°C. The eluate was re-amplified by direct PCR, using the same primers and the same PCR conditions as for the exponential PCR in NBS profiling. PCR products were checked on agarose gels and purified with Qiaquick PCR purification spin columns (Qiagen). Fragments were sequenced using an ABI 3700 automated sequencer (Applied Biosystems, USA). Several bands were also cloned using a pGem-T easy vector (Promega, USA), after a previous re-amplification. Four clones of each band were sequenced on an ABI 3100 automated sequencer (Applied Biosystems). Sequences from a same band displaying less than 95% homology were considered as distinct sequences. All the sequences of the amplified NBS-profiling-derived markers obtained were compared to GenBank sequence databases, using the blastx program (Altschul et al. 1997). The sequences displaying homologies with RGAs or R genes were aligned using the *PileUp* function of the GCG software package (Genetics Computer Group, Madison, Wis., USA).

Results

NBS profiling of the 'Discovery × TN10-8' progeny

Three degenerate primer/enzyme combinations were tested in the progeny: NBS2/*Mse*1, NBS2/*Rsa*1, and NBS3/*Mse*1. An average of 100 monomorphic bands per combination were obtained. Fifty-two polymorphic bands were identified with the three combinations and scored as dominant markers. Figure 2 shows a typical gel for NBS profiling. All these bands were mapped using the integrated linkage map. Fifteen markers displayed a band only in 'TN10-8', whereas 21 markers displayed a band only in 'Discovery'. These markers displayed a 1:1 segregation ratio. The 16 remaining markers displayed a band in both parents, with a 3:1 segregation ratio. Thirteen markers had a distorted segregation ($P > 0.05$) in the progeny, as assessed by chi-square tests, but were not discarded from genetic mapping. For clarity of discussion, NBS-profiling-derived markers are named NBS markers.
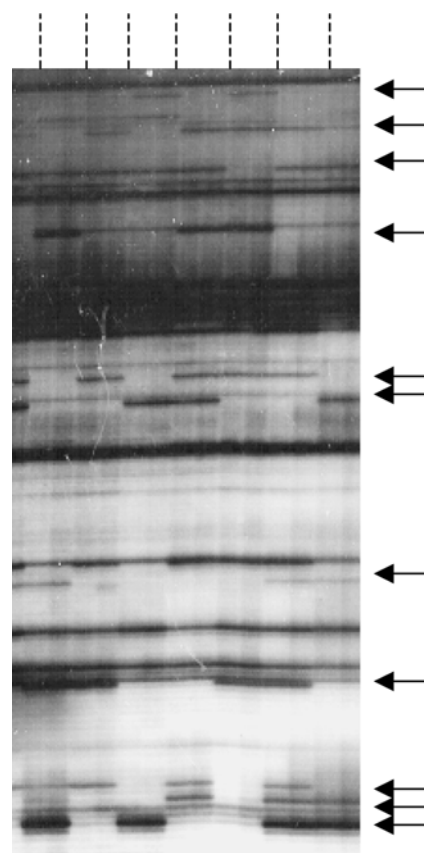
Fig. 2 Typical example of polymorphism revealed after electrophoresis by NBS profiling for six individuals belonging to the 'Discovery' × 'TN10-8' progeny (columns). *Arrows* indicate the positions of several polymorphic bands. This pattern was obtained with the *Mse*I enzyme and the NBS2 primer

Genetic mapping of the NBS-profiling derived markers (NBS markers)

Forty-three of the 52 polymorphic bands were reliably mapped either on the integrated linkage map or on the parental maps (Fig. 4). Three of the nine un-mapped markers could not be reliably assigned to a linkage group. The six remaining unmapped markers could be assigned to linkage groups with a high LOD score (LOD score > 5) but could not be mapped to linkage groups without changing markers order and genetic distances; each of these markers had a dis-torted segregation ratio. Eight markers could not be reliably mapped on the integrated linkage map, but could be mapped on the parental linkage maps. Markers were mapped to 10 of the 17 linkage groups of the apple genetic maps (Fig. 4). Most markers were organized in more or less wide clusters of 0–13 cM.

Sequence analysis of the NBS markers

Forty-one of the 52 polymorphic bands were excised from the polyacrylamide gels in order to be re-amplified and sequenced. The 11 remaining bands were not analysed, since it was impossible to excise them from the gels without excising surrounding bands. Each sequence contained the sequence of the NBS primer at their 3′ end. After comparisons with peptide sequences present in GenBank, it appeared that 22 out of the 41 sequences displayed homology with R genes or putative disease resistance proteins from different plant species (Table 1). Five sequences had homology with genes unrelated to disease resis-tance (results not shown). The 14 remaining sequences lacked homology with any peptide sequence.

Twenty-seven bands were also cloned before being sequenced. For 23 bands, two or more distinct se-quences sharing 50–80% homology were obtained. Seventy-five percent of these sequences were homolo-gous to RGAs or R genes. For only four of the cloned bands, all the sequences obtained were highly homologous (>95%) and further analysed as a single sequence. These four sequences displayed homologies with RGAs (Table 1).

Seventeen of the 22 sequences displaying homologies to RGAs (Table 1) could be aligned after translation (Fig. 3). Five sequences did not align with the other sequences, probably because of their high percentage of undetermined bases (11% on average). Two sets of sequences could be distinguished, containing either a 'VGIWG' or an 'ISVIPIVG' amino acid (aa) motif upstream the NBS primer (Fig. 3).

Table 1 Results of the blastx searches in peptidic sequences databases (GenBank) for the nucleotide-binding site (*NBS*)-profiling-derived markers. *LG* Linkage group, *Pos.* percentage of similar amino acids

Marker[a]	Length (bp)	LG[b]	Homology[c]	Species	E-value	Pos. (%)	GenBank accession no.
NBS2M01	398	T11	NBS/LRR-type disease resistance protein RPG1-B	*Glycine max*	3.6^E-02	64	AAR19096
NBS2M03	396	D2	TIR/NBS/LRR type R protein 7	*Malus baccata*	2.0^E-06	62	AAQ93075
NBS2M04	392	T2	TIR/NBS/LRR type R protein 7	*M. baccata*	3.0^E-08	83	AAQ93075
NBS2M07	332	D2	TIR/NBS/LRR type R protein 7	*M. baccata*	5.0^E-10	77	AAQ93075
NBS2M08	322	D17	TIR/NBS/LRR type R protein 7	*M. baccata*	3.0^E-03	70	AAQ93075
NBS2M09	307	D2	TIR/NBS/LRR type R protein 7	*M. baccata*	3.0^E-14	86	AAQ93075
NBS2M10	301	D2	TIR/NBS/LRR type R protein 7	*M. baccata*	2.0^E-06	69	AAQ93075
*NBS2M14	248	T12	Putative disease resistant protein RGA2	*Solanum bulbocastum*	2.6^E-01	53	AAP86601
NBS2M17	205	D2/T2	TIR/NBS/LRR type R protein 7	*M. baccata*	3.0^E-05	72	AAQ93075
NBS2R01	381	D8	NBS/LRR-type resistance protein	*Hordeum vulgare*	6.0^E-04	65	TO4389
*NBS2R09	251	D2	TIR/NBS/LRR type R protein 7	*M. baccata*	3.0^E-10	72	AAQ93075
NBS3M01	418	D11/ T11	Putative NBS/LRR disease resistance protein	*Arabidopsis thaliana*	2.0^E-03	68	NP_188065
NBS3M02	387	D2	TIR/NBS/LRR type R protein 7	*M. baccata*	5.0^E-03	54	AAQ93075
NBS3M03	382	T2	TIR/NBS/LRR type R protein 7	*M. baccata*	7.0^E-08	62	AAQ93075
*NBS3M03b	334	D17	Disease R protein NL25	*S. tuberosum*	1.0^E-04	66	CAA08797
NBS3M05	253	T10	Disease resistance protein homolog	*Brassica napus*	6.0e-04	59	AAK49082
NBS3M08	245	D10	Putative TIR/NBS type R protein 11	*M. domestica*	1.2^E-01	80	AAQ93077
NBS3M10	205	–	TIR/NBS/LRR type R protein 7	*M. baccata*	3.6^E-01	51	AAQ93075
*NBS3M12	208	T10	TIR/NBS/LRR type R protein 7	*Malus × domestica*	6.0^E-07	77	AAQ33077
NBS3M13	206	T10	TIR/NBS/LRR type R protein 11	*Malus × domestica*	5.0^E-05	75	AAQ93077
NBS3M15	158	–	TIR/NBS/LRR type R protein 7	*M. baccata*	5.0^E-12	86	AAQ93075
NBS3M16	140	D5/ T5	Putative disease resistance protein (TIR/NBS/LRR class)	*A. thaliana*	4.6^E-01	65	AAM15274

[a]Markers whose names are preceded by an asterisk were cloned before being sequenced. Only markers displaying homologies with resistance genes or resistance gene analogues are mentioned.

[b]*D* 'Discovery'; *T* 'TN10-8'
[c]*TIR* Toll/interleukin-1 receptor, *LRR* leucine-rich repeat

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A.
NBS2M1    RKFLRDWILLQRKKMSSI*K*VSPIEQR*DYXATSLVEDSSVYGREEDKERITKLLFSDDVIDDR ISVIPIVG MXGSGKN
NBS3M1    DEIXERLDFIAKEKDVLDLKIGVAHRTKMRLLATSLVEDSSVYGREEDEERITKLLLSDDVIDDR ISVIPIVG MPGSGKT
NBS2M14*  NKSIFD.SLTSHSKGYYDGHHSNLKHVIKARETHSFVRTSDVVGRGFEKEGIVHRLI..EQVDDH VSVIPVVG MPGSGKT
                                                                                  ◄────────
                                                                                  NBS primer


B.
NBS2M17   ~FRIP*FSGFEAXVLLEKLLTRLLRXWFPSTNEI.DVAKYPV.LESILSIQGITSHLSSGGSN.VLM VGIWG MXGSGKT
NBS2M9    MFTNSIFRR.RSKSYYEKLLTILLRKWLMSTNKL.RVAKHPVGINS..RIXXXISHLSSGGSN.VVM VGIWG MXGSGKT
NBS3M3    MLTNSIFR..REAKFIRXIVAXIITEWLPSTNEL.DVAKYPVGINS..RIQGITSHLSSGGSN.VLM VGIXX MXGSXKT
NBS3M12*  LDSQPESIDPIILN*ILPVRYEGISREL.NSNYL.DVARHPVGIDS..RVQEISNYLDVVGSNDVRV IGIWG MPGSGKT
NBS3M13   ~~~~~~~~LDPIILN*ILPIRYEGISREL.KSNYL.DVARHPVGIDS..RVQEISNYLD.VRSNDVRV IGIWG MPGSGKN
NBS2M7    LLSLPFQCFRREAKLIREIVDKIIPEWLPSAXKL.HVAKHPVGIYT..RIQDIITYLSNGGSDVVRM VGIWG MGGXGKT
NBS2R9*   LYFLTNCVFRHEAKLIKKIIYDNLWKWLPKTKKL.HVAKFPVGINS..RVQEIITYLSSGEGNKVLM VGIWG MPGSGKT
NBS3M10   ~~*XHNFSGREQXLLAVNCCTXLLRNGFQSTNEX.HVAKXP.GWNTIPRIQGIYHVIFQVDGSNVLM VGIWG MPGSGKN
NBS2M10   XQXMNSICQARSKSY*QKXLTILLRNGL*AXNXL.RVAKHPVGXNS..RIQDFFSHLSSDRSN.VVV VGIXG MXGLGKT
NBS2M4    CGRIQ..LXRREAKXIRXIXAKIIFXMAXRAQTN*YVAKDPVGINS..RIQGITSHLSXGGXN.VLM VGXGG XXGSXKT
NBS3M16   ~~~~~~~~~~~~~~~~~~~~~~~~*YXHIQTPIIIXATDTTVG.NGKARFEQIESSLCL.DSLDVYS IGIWG MPGSGKT
NBS3M3b*  FIYVVCSSCRYDTELIQGIVNTLWEKVH.RTFSMLDSSERFVG.T.DSKLEEIDLLLDT.DANDVRF IGIWG MRGSGKT
NBS3M5    MIDIYFLLHRYEGQFIQRIVHWVLNTLQH....VASDVEGLVG..IQSRVEELNRLLDI.ECYDVRF IGIWG MPGSXKT
NBS3M8    IEPFVSKYLTLCSNFRIELCTLIRFEGMQ*RTEVAIIWT*PYTQLE*ILACKKSVII*DVGSNDVRV IGIWG MXGSGKT
N         LKGSCDNRDKTDADCIRQIVDQISSKL...CKISLSYLQNIVG..IDTHLEKIESLlEI.GINGVRI MGIWG MGGVGKT
                                                                                  ◄────────
                                                                                  NBS primer
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Fig. 3 Amino acid (aa) alignment among translations of seventeen of the NBS sequences displaying homologies with resistance gene analogues. The aa sequences were aligned using the *PileUp* function of the GCG program. Only the last 80 aa of each sequence are shown. Two sets of sequences could be aligned, one with an 'ISVIPIVG' aa motif (**a**), the other with a 'VGIWG' aa motif (**b**) upstream the NBS domain. The second set of aa sequences (**b**) was aligned with the putative protein of the disease resistance gene *N* of tobacco, which contains a similar 'VGIWG' motif. Both motifs and the motifs corresponding to the NBS2/3 primers are *boxed*. *Asterisks* indicate a stop codon; *tildes* indicate an unknown sequence. Names of sequences that were cloned are followed by an *asterisk*

Discussion

The NBS-profiling method rapidly provides numerous polymorphic markers in apple

It was previously demonstrated that the NBS-profiling method allows the generation of polymorphic markers among different cultivars of potato, tomato, barley and lettuce (Van der Linden et al. 2004). The present study is the first one to test this new method in apple, and the first to demonstrate its utility for mapping studies using a segregating progeny. Fifty-two polymorphic markers were observed in the 'Discovery × TN10-8' progeny across the three enzyme/primer combinations tested, of which 43 could be mapped. Five out of the nine unmapped markers had a distorted segregation in the progeny, which could explain why they could not be reliably mapped. The P-loop motif targeted by degenerate primers used for NBS-profiling is known to be highly conserved through different plant species (Meyers et al. 1999). This is exemplified by the fact that, even though the primers were designed from R genes isolated in species not belonging to the Rosaceae family and PCR conditions were very specific, numerous bands could be obtained in apple that were putative RGAs.

Most of the NBS markers are RGAs

Twenty-three sequences displayed homologies with R genes or RGAs, though with rather weak similarities (high *E*-values) for some of them. The nine remaining sequences did not display homology to any peptide sequence. However, weak similarities, and even no similarity at all with any peptide sequence, does not necessarily indicate that these sequences are not RGAs. The NBS primers we used amplified DNA outside of the NBS domain, whereas the majority of the RGA sequences populating the sequence databases, including a number of apple RGAs (Lee et al. 2003), correspond to the NBS domain itself. Our NBS sequences therefore do not overlap with this population of RGAs. A number of R genes from different species have been fully sequenced, including the part that is targeted by the NBS primers we used. However, this region is only loosely conserved among different RGAs and species. High homologies in this region of RGAs can be expected in analogous genes of related species, or related R genes of apple, but only a few resistance genes or RGAs were previously identified in apple, and more generally in Rosaceae. This implies that sequences with high homologies to our apple NBS markers are rare.

The alignment of the NBS sequences obtained (Fig. 3) shows that most contained a 'VGIWG' aa motif upstream of the NBS primer. This is found as the P-loop motif in some Toll-interleukin-1 receptor/NBS/LRR-type RGAs such as those in *Arabidopsis thaliana* (Meyers et al. 2003). This means that the two degenerate primers used were actually anchored in the specific P-loop motif of the disease resistance proteins. This argument, together with those above, leads us to assume that at least the NBS markers displaying homologies

with R genes or RGAs after comparisons in databases are in fact RGAs. At least 23 of 41 markers, and possibly 32 (including the sequences with no homology with any peptide sequence), are assumed to be RGAs in the present study, i.e. 56% or more. This is comparable to the 50–90% previously found in several species (Van der Linden et al. 2004), the proportion varying according to the combination of species, primer and enzyme used.

Organization of the NBS markers in the apple genome

Most of the NBS markers were organized in more or less extended clusters. The most striking example is the cluster on linkage group (LG) 2, which includes 13 markers (Fig. 4). No recombination event was observed between NBS2M3 and NBS3M2 on LG D2, NBS2M4 and NBS3M3 on LG T2, NBS3M12 and NBS3M13 on LG T10, and NBS3M3b and NBS2M8 on LG D17 and T17. Therefore these markers correspond either to the same gene locus or to very tightly linked loci. The clustering of RGAs of the NBS/LRR type has previously been observed in numerous studies. Most NBS/LRR and other R gene-like sequences reside in large, extended arrays (Young 2000). This clustering of RGAs is not surprising, considering that plant R genes often, though not always, belong to gene families with evolutionarily related tandemly repeated genes, or to allelic series (Hulbert et al. 2001). The clustering of the markers mapped in this study adds to the evidence that most of them could be real RGAs.

Eighteen RGAs amplified from the NBS domain were already mapped in apple on a 'Fiesta' × 'Discovery' map (Baldi et al. 2004). Nine of these RGAs map in locations similar to those of our NBS markers on LG 1, LG 2, LG 5, LG 8, LG 10 and LG 17. Only the mapping of both sets of markers on the same genetic map might elucidate their precise relative positions. In the future, the identification of the NBS part of our markers would allow the determination of the similarity among markers belonging to both sets. The co-localizations of markers obtained through both methods, if confirmed, strengthen the probability of the existence of disease resistance gene clusters at these locations in the apple genome.

The sequences obtained here cannot be assigned with certainty to a NBS marker. This is illustrated by the fact that in most cases, several distinct sequences were obtained for those of the NBS markers that were cloned before being sequenced. It is highly probable that several distinct DNA fragments from NBS profiling may have the same molecular weight and cannot be easily distinguished. In addition, the re-amplification step before cloning might have led to an artificial increase in the concentration of some minor, artefact PCR products. In future experiments, it will be necessary to map the obtained sequences in order to confirm that they actually correspond to the expected marker. Such a study will allow the comparison of genetic and phylogenetic distances between markers.

High frequency of co-localization between NBS markers and loci for resistance to scab and mildew in apple

Twenty-five NBS markers out of 43 mapped in close vicinity to powdery mildew or scab major resistance genes previously identified in different progenies (Fig. 4). On LG 2, the major scab resistance gene *Vr2* co-segregated with the microsatellite marker CH02c02a in the apple progeny 'R12740-7A' × 'Idared' (Patocchi et al. 2003). According to the apple reference linkage map (Liebhard et al. 2003), this marker is located 4 cM below Ch02f06, which was mapped close to several NBS markers in our progeny. Baldi et al. (2004) also identified two NBS/LRR homologues between CH02c02a and CH02f06 (ARGH 37 and ARGH17). On LG 8, the major powdery mildew resistance gene *Pl-w* was mapped 13 cM below the microsatellite marker CH05a02 in a 'White Angel' × 'Gloster 69′ F_1 apple progeny (Evans and James 2003). Three NBS markers were mapped close to the putative position of *Pl-w* in our progeny. Baldi et al. also mapped a RGA on this LG (ARGH12), which might be located in the same region than ours if the orientation of their linkage map is correct. On LG 10, the major scab resistance gene *Vd*, previously identified in the apple cultivar 'Durello di Forli', was mapped in the apple F_1 progeny 'Durello di Forli' × 'Fiesta' 7 cM above the microsatellite marker CH02b07 (Tartarini et al. 2003). Four NBS markers mapped close to the putative position of *Vd* in our progeny. Once again, Baldi et al. (2004) also identified a RGA in this region (ARGH25), co-localizing with CH02b07. On LG 12, NBS3M11 was mapped at 0.7 cM from the scab resistance gene *Vg* in the 'Discovery' × 'TN10-8' F_1 progeny (Calenge et al. 2004). Since one recombinant was observed between them, NBS3M11 cannot correspond to *Vg* itself. For each of the four co-localizations mentioned, NBS markers could become useful makers for MAS or map-based cloning once converted to breeder-friendly markers and mapped in the appropriate progeny. For *Vr2*, *Pl-w* and *Vd*, NBS markers could provide candidate genes.

Several NBS markers also co-localized with QTL for resistance to scab and powdery mildew identified previously in the 'Discovery' × 'TN10-8' apple progeny (Calenge et al. 2003, 2004). Eleven NBS markers on LG 2, three NBS markers on LG 8, four on LG 10 and four on LG 17 were included in the confidence intervals of QTL for resistance to powdery mildew identified previously (Calenge et al. 2003). The co-localizations on LG 2, LG 8 and LG 10 also involved the major loci *Vr2*, *Pl-w* and *Vd*, respectively. On LG 10 and LG 17, Baldi et al. (2004) also identified RGAs in the regions covered by the QTL mentioned. In addition, an NBS marker mapped close to the likelihood peak of a strong effect QTL for resistance to two isolates of *V. inaequalis* on
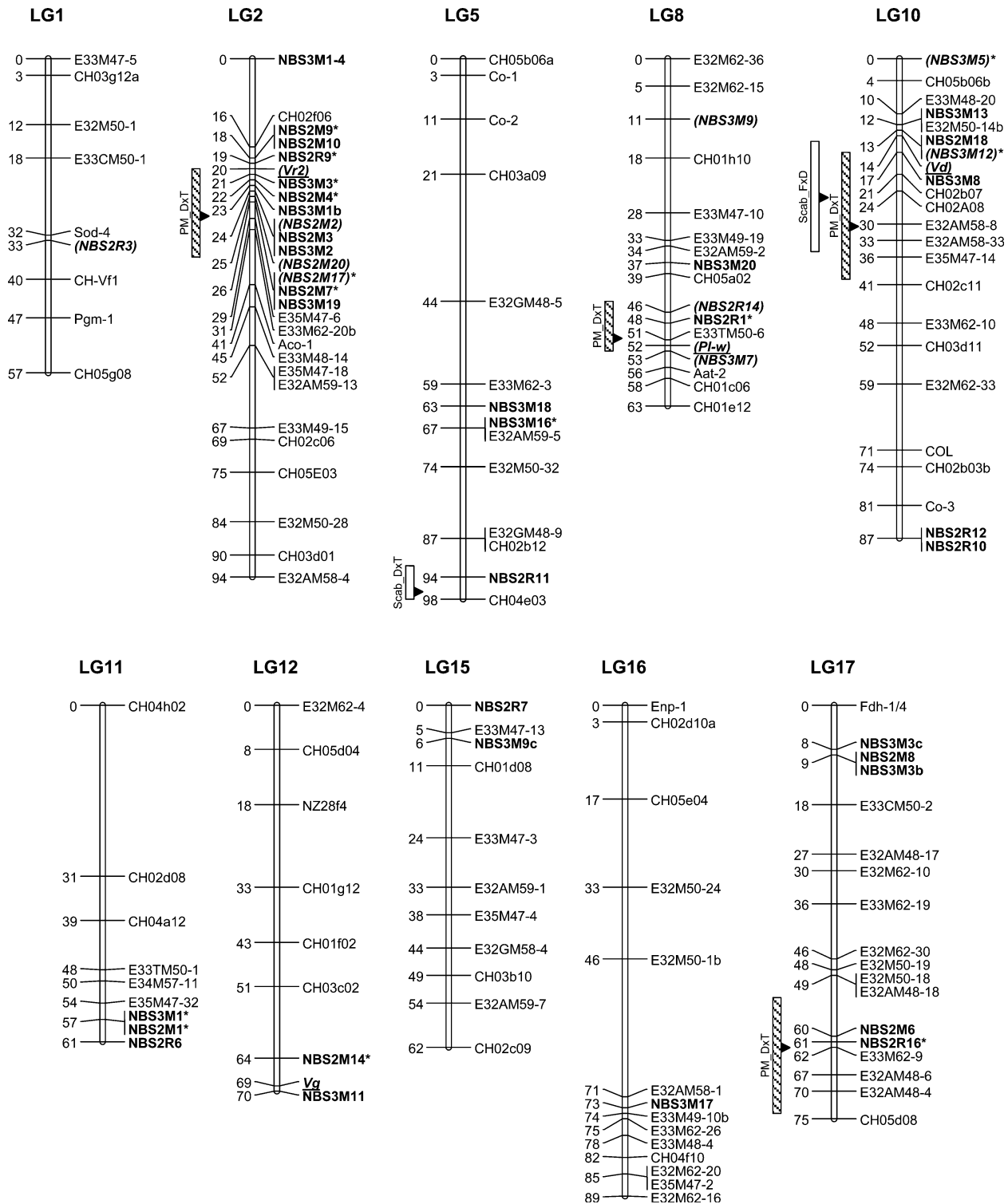
Fig. 4 Genetic positions (in centiMorgans) of the NBS-profiling-derived markers [(NBS markers) names in *boldface*] on the integrated linkage map of the apple F$_1$ progeny 'Discovery' × 'TN10-8' (D×T). Only linkage groups (*LG*) carrying NBS markers are shown. Names of the eight markers that could only be mapped on the separated parental linkage maps are *in brackets*. Names of major resistance genes mapped in other apple progenies are *underlined* and *in brackets*. Quantitative trait loci positions are represented by *rectangles* covering their confidence intervals. Names of NBS markers displaying homologies with functional or putative disease resistance proteins are followed by *asterisks*. *PM* Powdery mildew

LG 5 (Calenge et al. 2004). The NBS markers co-localizing with QTL might prove useful for MAS. Proving that they actually are the gene(s) underlying QTL would require a tremendous amount of work, due to the large confidence intervals surrounding QTL and to their weak effects compared to major genes. In addition, pseudog-

enes are very frequent among NBS/LRR-like genes and are expected to display much more polymorphism than functional genes, so that they are often assumed to be the main source of variation. Therefore, RGAs are more useful as landing markers than as candidate genes. Co-localization among RGAs and QTL have previously been observed in a number of species, including bean (Geffroy et al. 2000), soybean (Kanazin et al. 1996), lettuce (Shen et al. 1998), *A. thaliana* (Speulman et al. 1998), maize (Collins et al. 1998) and barley (Backes et al. 2003). These co-localizations raise the possibility that some of the genes underlying quantitative resistance share structural and functional similarities with R genes.

Not all QTL for resistance to scab and mildew co-localized with NBS markers. This result is not surprising because (1) the method used did not allow the mapping of all NBS-containing DNA fragments, as revealed by the high number of monomorphic bands in the progeny; (2) not all classes of R genes were targeted; and (3) not all of the QTL for resistance are expected to be related to R genes. Several studies reported the occurrence of genetic co-localizations among defence-related genes and QTL for resistance (e.g. Faris et al. 1999; Gebhardt and Valkonen 2001; Pflieger et al. 2001; Trognitz et al. 2002; Ramalingam et al. 2003). Therefore, some of the plant disease resistance QTL may be involved in general defence pathways. On the other hand, not all NBS markers co-localized with known resistance loci. These markers may (1) not be homologous to R genes, or correspond to R gene-like sequences that evolved to confer functions other than disease resistance; (2) belong to isolated pseudogenes; or (3) correspond or map close to functional but so far undetected resistance loci. Some of those undetected loci might belong to the large range of overcome, 'ephemeral' resistance genes which have been postulated in apple (MacHardy et al. 2001). Provided that the mapped NBS markers are real RGAs, they could predict the existence of functional resistance genes or QTL, which have been undetected so far. For instance, the presence of several NBS markers clustering on LG 11 could be an indication that there are functional resistance loci at this genetic position.

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