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M.R. Marano · I. Malcuit · W. De Jong
D.C. Baulcombe

High-resolution genetic map of *Nb*, a gene that confers hypersensitive resistance to potato virus X in *Solanum tuberosum*

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Abstract *Nb* is a single dominant gene in potato that confers hypersensitive resistance to potato virus X (PVX) isolates from strain groups 1 and 2. Genetic and molecular analyses showed that *Nb* is located on the upper arm of chromosome V and forms part of a cluster of resistance genes encoding specificities to many different pathogens. We describe the genetical localisation of molecular markers tightly linked to the *Nb* locus and the development PCR-based markers suitable for isolation of the *Nb* resistance gene by positional cloning. A bulked segregant approach was applied to identify polymorphic AFLP markers tightly linked to the *Nb* locus. These markers were mapped in a population of segregating S1 progeny (1,300 plants) from a self-pollinated potato cultivar, Pentland Ivory. From this analysis, *Nb* was placed in an interval of 0.76 cM, flanked by the AFLP markers GM339 and GM637. Recombinant PVX strains carrying different combinations of avirulence genes were used in biological assays to show that *Nb* was also present in potato cv. Cara but was masked by the extreme PVX resistance conferred by the *Rx* gene. PCR-based screening of a Cara genomic BAC library with markers closest to the *Nb* locus identified a new marker tightly linked to *Nb*.

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M.R. Marano and I. Malcuit contributed equally to this work

M.R. Marano · I. Malcuit · W. De Jong · D.C. Baulcombe (✉)
The Sainsbury Laboratory, John Innes Centre, Colney Lane, Norwich, NR4 7UH, UK
e-mail: baulcombe@sainsbury_laboratory.ac.uk
Fax: +44-1603-450011

Present addresses:
M.R. Marano, Instituto de Biologia Molecular y Celular de Rosario (CONICET), Departamento Microbiologia, Facultad de Ciencias Bioquímicas y Farmacéuticas, Universidad Nacional de Rosario, Suipacha 531, 2000 Rosario, Argentina

W. De Jong, Department of Plant Breeding, 405 Bradfield Hall, Cornell University, Ithaca, NY 14853, USA

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Introduction

Genetic control of resistance to pathogens in plants is often determined by simple gene-for-gene interactions. The resistance response is induced only if the pathogen encodes a strain-specific avirulence (*avr*) gene and the plant carries the corresponding disease resistance (*R*) gene (Flor 1971; Keen 1990). It has been proposed that *R* genes are members of multigene families encoding putative receptors that recognise specific molecules (ligands) derived directly or indirectly from pathogen *avr* genes. In many cases, this recognition is manifested as a hypersensitive response (HR) which is associated with a programmed cell death at the initial site of infection (Morel and Dangl 1997).

Over the last decade more than 20 *R* genes against pathogens as diverse as viruses, bacteria, fungi, insects and nematodes have been isolated by map-based cloning or transposon tagging in different plant species (reviewed by Meyers et al. 1999). It is currently unknown how many different types of *R* genes there are in plants. Analysis of the predicted protein sequences of these genes revealed that the most common types are proteins containing a nucleotide binding site (NBS) and leucine-rich repeats (LRRs) of various lengths which are potentially a ligand-specific recognition domain (reviewed by Ellis et al. 2000). These proteins can be divided into two major classes, depending on the presence or absence of an amino terminal toll/interleukin-1 like receptor motif (TIR) (Michelmore 2000). In the non-TIR class of proteins, the TIR domain is often replaced by a coiled-coiled (CC) structure, which in some cases has the features of a leucine zipper (LZ) (Young 2000). It is thought that the TIR and CC/LZ motifs are involved in signal transduction (Young 2000). These conserved structural motifs in R proteins suggest that plants have common

signal transduction pathways for different defense responses. However, the molecular basis of the *R-avr* recognition and the signalling programme activated immediately following this event is still largely unknown.

Two types of resistance to potato virus X (PVX) have been identified in *Solanum tuberosum*: hypersensitive resistance controlled by the genes *Nb* and *Nx* and extreme resistance (ER) conferred by the *Rx1* and *Rx2* genes. *Rx1* and *Rx2* are located on chromosome XII and V, respectively (Bendahmane et al. 1997; Ritter et al. 1991), and belong to the CC/LZ-NBS-LRR class of resistance genes (Bendahmane et al. 1999, 2000). *Nx* maps to chromosome IX (Tommiska et al. 1998), and *Nb* is located on chromosome V (De Jong et al. 1997) in a region where other loci, (*Rx2*, *R1*, *Gpa*, *Gpr1*) conferring resistance to many different pathogens have been mapped (Ritter et al. 1991; Kreike et al. 1994; Leonards-Schippers et al. 1992; Roupe van der Voort et al. 1998). For two of the *R* genes present in this region, the corresponding pathogen *avr* gene has been characterised. Induction of the *Nb*-mediated HR in potato cells is dependent upon the expression of the PVX 25-kDa viral movement protein (Malcuit et al. 1999), whereas the *Rx2*-mediated resistance is elicited by the viral coat protein (Bendahmane et al. 1995). In this paper we describe the localisation of molecular markers tightly linked to the *Nb* locus from potato cv. Pentland Ivory. These markers were generated using amplified fragment length polymorphism (AFLP) technology and during the screening of a potato BAC library from potato cv. Cara. The development of polymerase chain reaction (PCR) based markers suitable for the detection of *Nb* in practical breeding programmes will be useful for isolation of this gene by positional cloning and the genetical characterisation of the cluster of *R* genes on chromosome V.

Materials and methods

Plant material, virus strains, PVX cDNA clones and resistance assays

Tetraploid potato cv. Pentland Ivory carrying *Nb* in the simplex condition (*Nb nb nb nb*) (De Jong et al. 1997) was self-pollinated to produce an S1 population. Tetraploid potato cv. Cara carrying *Rx1* in duplex condition (*Rx1 Rx1 rx1 rx1*) has been described previously (Kanyuka et al. 1999).

PVX strains ROTH1, CP2, UK3 and HB have been described previously (Malcuit et al. 1999; Orman et al. 1990; Kavanagh et al. 1992; Querci et al. 1993). PVX strains ROTH1, CP2 and UK3 belong to PVX strain groups 1, 2 and 3, respectively, based on the classification of Cockerham (1955). The plasmid pU1HB (Fig. 3) was constructed by substituting the *Apa*I-*Avr*II fragment from pUK3-Ile6 (Malcuit et al. 1999), which contains an avirulent version of the 25-kDa protein, into the Ti plasmid vector pGR103 (R. Lu, unpublished data), which carries the genomic sequence of UK3 with a coat protein derived from PVX strain HB (Fig. 3). The PVX cDNA is under the control of the CaMV 35S promoter.

Plants were tested for resistance to PVX avirulent strains by graft inoculation (De Jong et al. 1997) and/or particle bombardment of detached leaves (Malcuit et al. 1999; Marano and Baulcombe 1998). Plants were considered resistant (*Nb*) if they displayed HR (necrotic lesions) on leaves inoculated with PVX avirulent strains ROTH1 or CP2 and if no virus was detected on systemic leaves. Plants were considered susceptible (*nb*) if after challenging with ROTH1 or CP2 the plants became systemically infected with the virus. To monitor virus accumulation in infected plants, we carried out ELISA tests with polyclonal (ELISA kit for detection of PVX; Boehringer, Mannheim) and monoclonal antibodies MAC58 or MAC72 (Torrance et al. 1986) prepared against the PVX coat protein.

Histological assay

Aniline blue staining for the presence of callose deposition in potato leaves undergoing HR was performed as described previously (Dietrich et al. 1994). Leaves were examined and photographed under UV light with a Zeiss Axiophot epifluorescence microscope.

PCR-based screening of the mapping population and AFLP analysis

Genomic DNA for AFLP and PCR-based analyses was isolated according to Bendahmane et al. (1997). Primers, PCR conditions and restriction enzymes used for the markers GP21, SPUD839, TG432 and SPUD237 have been described previously (De Jong et al. 1997). To isolate DNA for the resistant (R) and susceptible (S) bulked segregant pools, equivalent amounts of leaf material from individuals in each class were pooled before extraction (Michelmore et al. 1991). Template DNA from the R and S pools as well as from each individual recombinant plant was prepared for AFLP as described previously (Thomas et al. 1995; Vos et al. 1995), using the restriction enzymes *Pst*I and *Mse*I. For selective amplification, 741 primer combinations were analysed: 13 *Pst*I/+2 primers (two selective nucleotides) and 57 *Mse*I/+3 primers (three selective nucleotides). AFLP reactions were performed essentially as described by Thomas et al. (1995). Selective amplification products were separated on a 4.5% polyacrylamide sequencing gel run at 100 W for 2.5 h. After electrophoresis, gels were vacuum-dried onto 3 MM Whatman paper (Whatman, Maidstone, UK) and exposed to X-ray film (BioMax MR, Kodak) overnight. Bands of interest were cut out of the gel with a scalpel and incubated in 150 μl of TE (10 m*M* Tris pH 7.5, 1 m*M* EDTA pH 8.0) overnight

Table 1 PCR-based markers linked to the *Nb* locus. For each marker, the sequence of the primers and the PCR conditions are shown

Marker[a]	Primer (5′→3′)	PCR conditions[b] (35 cycles)
GM339	GGT AGT TGG ACG AGC ATA T CTC ACT TTT AGA CCA GAT TT	94 °C,15 s; 52 °C, 15 s; 72 °C, 40 s
GW339	GGC TGG TAC TAA CAG CAA GCC AAT GGG CTG GTA TTC AAA AAT CGT CTC	94 °C, 20 s; 52 °C,15 s; 72 °C, 60 s
GM637	GCA GAA GAT CGG ATA GCA AAC GTA ACG AGT TGA AGT TAC TGA	94 °C, 15 s; 55 °C, 15 s; 72 °C, 40 s
98L	GAA TGA TGA AGA AAG ACA TC GTT AAG GAC ACA TAT ATG AC	94 °C, 15 s; 52 °C, 15 s; 72 °C, 50 s

[a] Allele-specific markers (i.e. primer sets only amplify the allele linked in *cis* to *Nb*)
[b] All PCR reactions were started with an initial denaturation step of 2 min at 94 °C

at 37 °C. AFLP fragments were recovered by PCR using the same conditions as the initial amplification. PCR products were cloned into the pGEM-T vector (Promega, Madison, Wis.). To confirm that the cloned DNA was the same size as the original AFLP marker, we carried out an AFLP reaction on the plasmid DNA. The products were fractionated by electrophoresis alongside the original selective amplification products from the bulked segregants. Cloned AFLP markers were then sequenced and converted to PCR-based markers. The sequences and PCR conditions used for derivatives of AFLP markers are shown in Table 1.

Inverse PCR for genome walking

An uncloned library prepared from potato DNA for genome walking by PCR was constructed using the Universal GenomeWalker kit according to the manufacturer's instructions (Clonetech, Palo Alto, Calif.). For the construction of each library, 2.5 μgof genomic DNA from the R pool was digested with five different restriction enzymes (*Dra*I, *Eco*RV, *Pvu*II, *Sca*I, *Stu*I). After phenol extraction, the DNA was ethanol-precipitated, resuspended in 20 μl of TE and ligated to genome walker adaptors with 0.5 U of T4 DNA ligase (Boehringer, Mannheim) at 4 °C overnight. This DNA library was used as a template in a primary PCR reaction using the AP1 primer and primers designed based on the ends of the marker GM339. An aliquot of this PCR product was used as template in a second PCR reaction with the AP2 primer and "nested" primers designed based on the ends of the GM339 sequence. The PCR products obtained were cloned directly into the pGEM-T vector and sequenced. AP1 and AP2 primers were provided with the kit.

Sequencing of PCR products and BAC ends

PCR products were cloned into pGEM-T, amplified using M13 universal and reverse primers (Amersham Pharmacia Biotech) and purified using the QIAquick PCR extraction kit (QIAGEN, Valencia, Calif.). The right and left end-sequences of insert DNA from the Cara BAC library were isolated by inverse PCR (IPCR) according to Kanyuka et al. (1999). Sequencing reactions were carried out using the ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kit (Perkin Elmer, Foster city, Calif.) according to the manufacturer's instructions. Database searches were performed using the basic local alignment search tool (BLAST; Altschul et al. 1990).

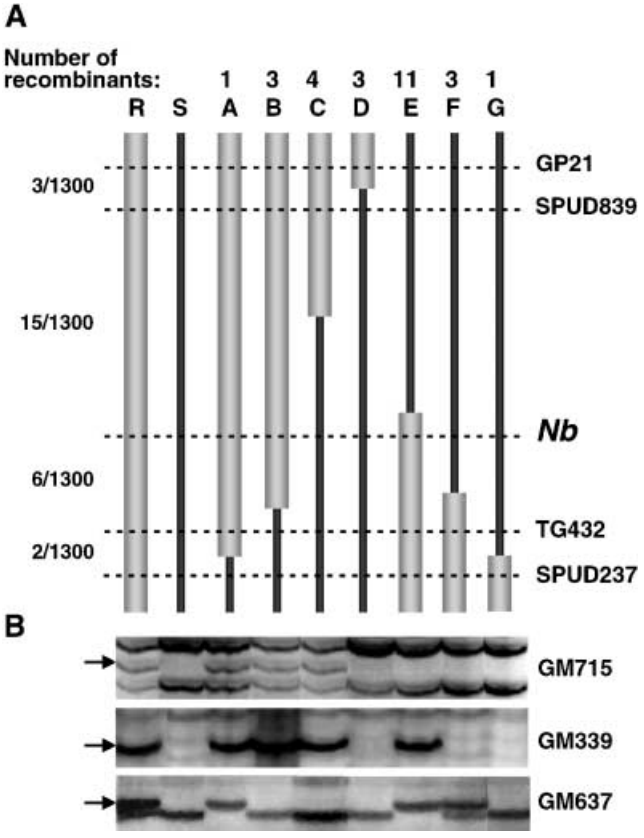

Fig. 1A, B Segregation patterns observed for markers closely linked to *Nb*. **A** Schematic representation of recombinant classes identified around the *Nb* locus (*A–G*). The resistant (*R, solid bar*) and susceptible (*S, black line*) genotypes are shown. Markers defining recombination sites are indicated on the *right*. The number of recombinants plants (from a total population of 1,300) belonging to a particular class are shown on the *left*. The number of AFLP markers identified for each interval is also shown. **B** Examples of AFLP markers (one of each class) identified in the intervals indicated in *A*. Lanes correspond to the recombinant clases shown in *A* (*R, S, A, B, C, D, E, F* and *G*). The name of the AFLP markers is shown on the *right*. *Arrows* indicate the linked AFLP fragment to *Nb*

Results

Identification of recombination events in the GP21-SPUD237 interval

De Jong et al. (1997) was previously mapped *Nb* on chromosome V in a 3.3-cM interval delimited by markers GP21/SPUD839 and TG432/SPUD237. We extended the original mapping population to a total of 1,300 S1 progeny to allow the construction of a high-resolution map around the *Nb* locus. This S1 population was screened for recombination events using PCR analysis of DNA samples from individual plants with primers derived from GP21 and SPUD237 markers as described previously (De Jong et al. 1997). The segregation of polymorphism for both markers was 3:1 (971 plants from a total population of 1,300 S1 individuals), which is consistent with *Nb* being in simplex condition in cv. Pentland Ivory. Twenty-six individual plants were identified with recombination events in the interval between markers GP21 and SPUD237. The markers SPUD839 and TG432 initially co-segregated with GP21 and SPUD237, respectively (De Jong et al. 1997). After screening of the recombinant plants, these markers were separated from GP21 and SPUD237 by three and two recombination events, respectively (Fig. 1A). Recombinant plants were then tested for resistance to PVX-avirulent strains CP2 or ROTH1 and divided into seven classes according to their resistance/susceptibility phenotype and the molecular pattern defined by each AFLP marker (Fig. 1A). *Nb* was mapped between SPUD839 and TG432 at 1.15 cM and 0.46 cM, respectively (Fig. 1A).

High-resolution genetic map around the *Nb* locus

A high density genetic map of the region carrying *Nb* was required to facilitate the positional cloning of this gene.

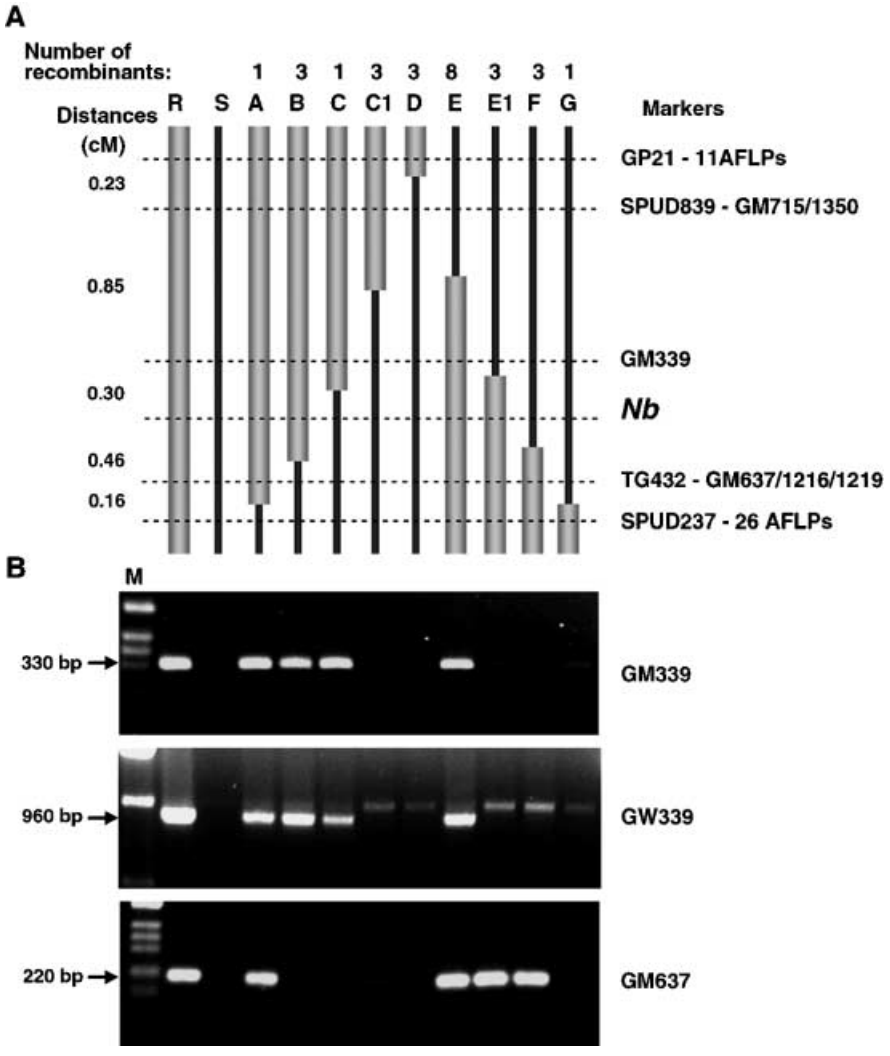

Fig. 2A, B High-resolution genetic map of the *Nb* locus. **A** Map position of the *Nb* locus based on the screening of 1,300 segregant plants with AFLP markers linked to the *Nb* locus in the GP21-SPUD237 interval. The genetic distance (centiMorgan, cM) corresponds to the percentage of recombination between molecular markers or molecular markers and *Nb* in a population of 1,300 plants. **B** Shows the analysis of individual plants [resistant to PVX-ROTH1 (*R*) or susceptible (*S*)] with recombination events in the GP21-SPUD237 interval using the GM339 and GM637 PCR-based markers. The *R* pool and *S* pool are indicated as *R* and *S* respectively. *Arrows* show the positions of the molecular markers

To identify DNA markers in the region delimited by the markers SPUD839 and TG432 (containing *Nb*), AFLP technology (Vos et al. 1995) was employed in conjunction with a bulked segregant approach (Michelmore et al. 1991). The AFLP analysis was carried out on DNA pooled from 20 R and 20 S plants selected from the S1 progeny of potato cv. Pentland Ivory. A total of 741 randomly selected *Pst*I/*Mse*I primer combinations were analysed. Each AFLP primer combination displayed, on average, 100 DNA fragments so that approximately 74,100 loci were evaluated for polymorphism, from which 69 primer combinations revealed polymorphisms between the R and S pools. To map these new polymorphic markers relative to SPUD839 and TG432, we repeated the AFLP analysis using the recombinants detected in the GP21 and SPUD237 interval. Thus, DNA samples from recombinant plants of classes A, B, C, D, E, F and G (Fig. 1A) were assayed with the 69 informative primer combinations. Of these, 11 AFLP markers co-segregated with GP21, six AFLP markers (GM715, GM1350, GM339, GM637, GM1216 and GM1219) were mapped in the interval defined by SPUD839 and TG432 (Table 2, Figs. 1B, 2A), 26 AFLP markers co-segregated with SPUD237 and the remaining 26 markers were mapped outside the GP21-SPUD237 interval.

Table 2 Selective bases for amplification of AFLP loci linked to *Nb* in potato

Marker	AFLP primers *Pst*I+2/*Mse*I+3
GM715	CC/GTA
GM1350	TG/TAT
GM339	GG/CTC
GM1216	GT/GTT
GM1219	GT/CTT
GM637	AA/CGA

In the second step, the AFLP markers GM715, GM1350, GM339, GM637, GM1216 and GM1219 were assigned to more precise chromosomal positions by AFLP analysis of individuals of each class of recombinant plants. GM715 and GM1350 co-segregated with SPUD839. GM339 mapped between SPUD839 and *Nb* at 11 and four recombination events, respectively. The other three markers (GM637, GM1216 and GM1219) co-segregated with TG432 (Fig. 2A). Results from this

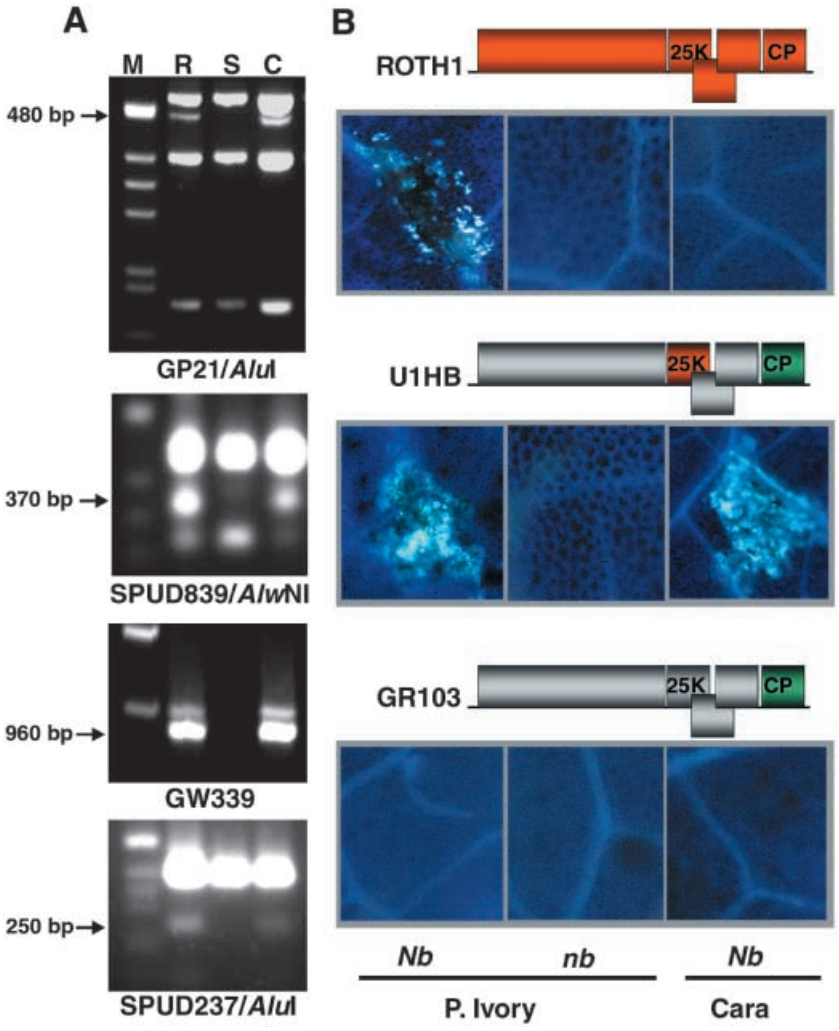

Fig. 3A, B Molecular and biological assays for the presence of *Nb* in Cara. **A** Analysis of genomic DNA from potato cv. Pentland Ivory (*R*) and (*S*) pools and potato cv. Cara (*C*) with markers GP21, SPUD839, GW339 and SPUD237. The PCR products were digested with *Alu*I for the GP21 and SPUD237 markers and with *Alw*N1 for the SPUD839 marker. The DNA fragments linked in *cis* to the *Nb* locus are marked by an *arrow*. **B** Pentland Ivory resistant (*Nb*) and susceptible (*nb*) leaves and Cara leaves were inoculated by particle bombardment with the PVX avirulent strain ROTH1 and the avirulent (U1HB) or the virulent (RG103) PVX hybrid. Leaves were stained with aniline blue at 4 days post-inoculation to detect callose deposition as an indicator of HR

analysis place *Nb* in an interval of 0.76 cM, flanked by the marker GM339 and the cosegregating markers TG432, GM637, GM715 and GM1219 (Fig. 2A). To develop a fast screening method, the AFLP markers closest to *Nb*, GM339 and GM637, were converted to PCR markers. GM339-derived primers amplified a 330-bp fragment and GM637-derived primers, a 220-bp fragment (Fig. 2B). The GM339 and GM637 primers amplified only the resistant allele and were used to analyse the 26 plants with recombination events between GP21 and SPUD237. The results obtained from this analysis were in accordance with the map presented in Fig. 2A.

The presence of repeated DNA sequences has been shown to cause allele-specific primers to identify BAC clones from the susceptible chromosome or from non-allelic sites. Presumably, this artefact results from the high abundance of primer-binding sites in the cloned DNA (Kanyuka et al. 1999). Therefore, to confirm our results we carried out Southern analysis on the DNA of the R and S pools digested with restriction enzymes *Hin*dIII, *Eco*RI and *Eco*RV using markers GM339 and GM637. The results showed that each marker was present as a single copy and exclusively in the resistant pool (data not shown).

Resistance test for the presence of *Nb* in potato cv. Cara

Potato cvs. Pentland Ivory and Cara share part of their genetic background (Cockerham 1955; Hawkes 1994). To find out whether this common background includes *Nb*, we carried out PCR analysis with GP21, SPUD839, GM339, TG432, GM637 and SPUD237. In each instance, the amplified fragments from cv. Cara genomic DNA showed the same restriction pattern as the equivalent fragments of cv. Pentland Ivory (Fig. 3A; data not shown). Moreover, the nucleotide sequence of the two allele-specific markers GM339 and GM637 were identical in both cultivars, and it seemed likely that *Nb* could be present in cv. Cara. However, we could not test this hypothesis because Rx1 protects cv. Cara from most natural strains of PVX. If both *Nb* and *Rx*1 resistance genes are present in cv. Cara, the *Nb*-mediated response would be masked by the extreme resistance response conferred by *Rx1* (Cockerham 1970). Therefore, to find out whether cv. Cara carries a functional *Nb* we carried out a series of tests with recombinant PVX isolates that were based on the naturally occuring PVX strains UK3, HB and ROTH1. The design of the recombinant isolates exploited our previous finding that the coat protein in UK3 is an *Rx*

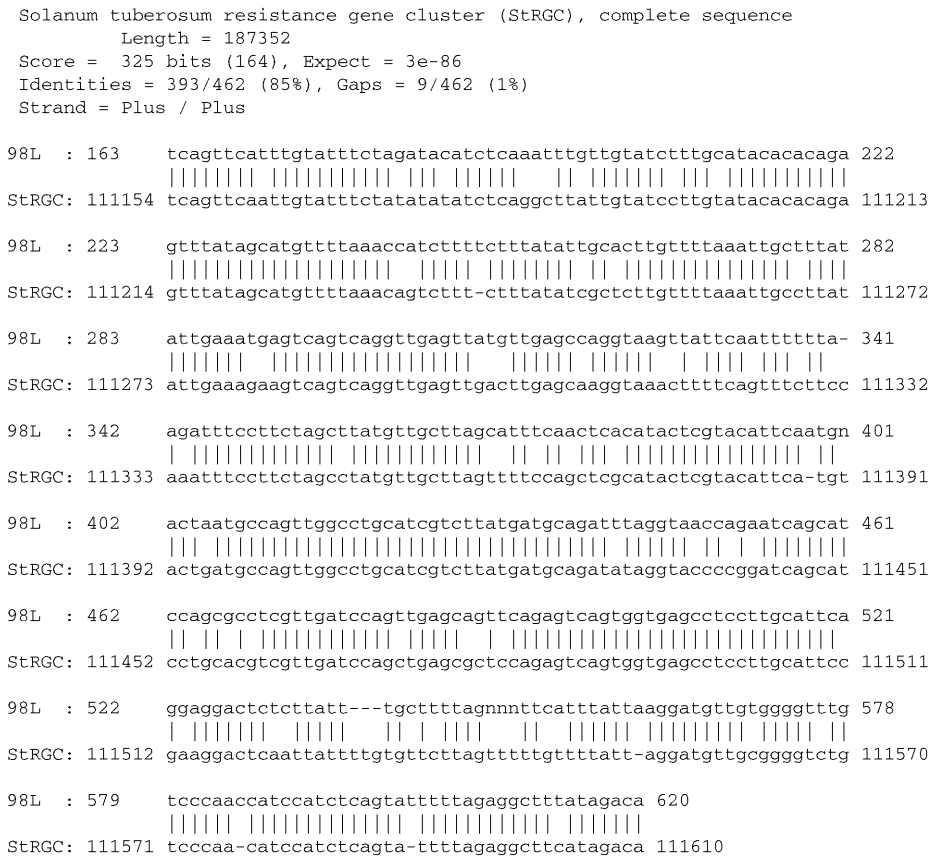

Fig. 4 Sequence analysis of marker 98L The nucleotide sequence alignment of marker 98L with a sequence corresponding to a cluster of resistance genes on chromosome XII (StRGC, Van der Vossen et al. 2000) was produced with the BLAST programme (Altschul et al. 1990)

avirulence determinant (Bendhamane et al. 1995) and that the 25 kDa protein in ROTH1 is an *Nb* avirulence determinant (Malcuit et al. 1999). Thus, we examined the recombinant PVX isolate GR103 (R. Lu, unpublished data) that is based on the UK3 isolate but with a coat protein derived from the *Rx* resistance-breaking strain HB (Fig. 3B). A second recombinant PVX isolate, U1HB, which is based on GR103 but with the *Nb* avirulence- determining 25-kDa protein derived from ROTH1 (Fig. 3B). GR103 is able to overcome resistance specified by both *Rx* and *Nb*, U1HB is able to induce *Nb*-mediated HR but not *Rx*1-mediated extreme resistance and the natural isolate ROTH1 is recognised by plants carrying both resistance genes.

To test the presence of *Nb* in cv. Cara, we carried out particle bombardment of detached leaves (Malcuit et al. 1999) to inoculate PVX, followed by aniline blue staining for callose deposition as an indicator of HR (Fig. 3B). HR lesions developed on cvs. Cara- and Pentland Ivory-resistant (*Nb*) leaves bombarded with U1HB, whereas leaves bombarded with GR103 did not (Fig. 3B). Cultivar Cara leaves bombarded with ROTH1 did not develop HR lesions, whereas cv. Pentland Ivory leaves did (Fig. 3B). These results indicate that *Nb* is present in cv. Cara and that *Rx1* is epistatic to *Nb*.

Identification of new markers linked to *Nb* derived from a cv. Cara BAC clone

To identify more markers in the vicinity of *Nb*, we analysed a potato BAC library prepared from cv. Cara (Kanyuka et al. 1999). The screening of this library with markers tightly linked to *Nb* identified several positive BAC pools with GP21, SPUD839, TG432, GM637 and SPUD237 (data not shown).

One of the positive BAC clones identified (BAC98) was found to carry the TG432 marker and allele-specific marker GM637 within a genomic DNA insert of 120 kb. *Rsa*I digestion of the TG432 PCR product revealed that BAC98 carried the TG432 allele linked in *cis* to *Nb* (data not shown). This BAC was selected as a potential source of markers linked to *Nb*.

To determine the end sequence of BAC98, we isolated its left (L) and right (R) ends by IPCR (Kanyuka et al. 1999), determined their nucleotide sequences and produced primers for the PCR-based markers 98L and 98R. The 98L marker amplified one band of 650 bp in BAC98 linked in *cis* to *Nb* (Fig. 4). Sequence analysis of this marker revealed that 98L from cvs. Cara and Pentland Ivory were identical. Analysis of the cv. Pentland Ivory recombinant plants with this marker showed that 98L cosegregated with GM637 and TG432. The 98R marker amplified BAC35 and BAC195, which also contained SPUD237 (data not shown). Further PCR screening of the cv. Cara BAC library with 98L and GM339 failed to

identify a BAC clone that spanned the gap between both markers.

As GM339 is only 330 bp in length, we used a genome walking strategy (see Materials and methods) to clone its flanking sequences. The resulting new marker, GW339, was 960 bp in length (Fig. 2B) and was used to re-screen the cv. Cara BAC library. This improved marker also failed to identify any BAC clone that would close the gap between GM339 and 98L.

Interestingly, the marker 98L shows homology in parts to a sequence in a region of the potato genome corresponding to a cluster of resistance genes from chromosome XII (Van der Vossen et al. 2000) (Fig. 4).

Discussion

In this report we describe new molecular markers tightly linked to the *Nb* locus. As part of the strategy for cloning *Nb*, a large number of segregating S1 progeny (1,300 plants) from a self-pollinated potato cultivar, Pentland Ivory, has been screened, and a high-resolution genetic map was constructed around the *Nb* locus using AFLP technology (Vos et al. 1995) in conjunction with a bulked segregant approach (Michelmore et al. 1991). A total of 69 *Pst*I/*Mse*I primer combinations showed polymorphism between the R and S pools. From these, only six were found in the interval between DNA markers GP21 and SPUD237, spanning a region of 2 cM, whereas 11 and 26 AFLP markers co-segregated with GP21 and SPUD237, respectively, indicating that there is a distorsion in the distribution of markers in this region. The lower degree of polymorphism in this interval could be due to the fact that *Pst*I recognises a GC-rich hexanucleotide sequence that could be subjected to methylation (McClelland et al. 1994). Therefore, this methylation-sensitive enzyme would cleave preferentially in transcribed regions that are normally hypo-methylated and where the level of sequence conservation is high (Antequera and Bird 1988). Like most cultivated species from the *S. tuberosum* ssp. *tuberosum* group, Pentland Ivory was derived from *S. tuberosum* ssp. *andigena* that originally carried the *Nb* gene (Jones 1981; Ross 1986). Previous studies have shown that there is variation in the frequency of recombination in areas that have been introgressed from alien species (Kanyuka et al. 1999, Liharska et al. 1996). Therefore, the low level of polymorphism between GP21 and SPUD237 could also be explained by the presence of introgressed DNA from *S. tuberosum* ssp. *andigena* into cv. Pentland Ivory.

In the high-resolution genetical map described above, we positioned the *Nb* locus in an interval of about 0.76 cM between the AFLP markers GM339 and GM637 (Fig. 2A). Given that the average recombination frequency in potato is about 1,000 kb cM^{-1} (Tanksley et al. 1992), the distance between the closest flanking markers would correspond to approximately 760 kb. However, previous studies in potato have shown that the relationship between genetical and physical distances can vary considerably. For instance, in the case of potato cv. Cara, which was used to isolate the *Rx1* gene, recombination frequencies were found to vary from 180 kb cM^{-1} to 2,677 kb cM^{-1}, estimated from the number of recombination events in individual BAC clones (Kanyuka et al. 1999). Therefore, the physical distance in the genetical interval between markers GM339 and GM637 cannot be estimated accurately at this stage. However, the small number of AFLP markers identified near the GP21 and SPUD237 interval and their resistant-allele specificity may indicate that the physical distance in this genetic interval is small.

Here we show that *Nb* is present in cv. Cara using a biological assay with a recombinant virus derived from the *Rx* resistance-breaking strain PVX-HB that carries the avirulent 25-kDa protein that is recognised by *Nb* (Malcuit et al. 1999). Screening of the cv. Cara BAC library identified one clone containing the co-segregating markers TG432 and GM637 from the resistant allele within a genomic insert of 120 kb. A new resistant allele-specific marker, 98L, was generated from the left end of this BAC clone. In addition, we found that *Nb*-flanking markers GM339, GM637 and 98L were identical at the nucleotide level in both cvs Cara and Pentland Ivory. The likely explanation for the presence of *Nb* in both cultivars is that *S. tuberosum* ssp. *andigena* was used in breeding programmes to introgress pathogen resistance into cultivated potato cultivars, including cvs. Cara and Pentland Ivory (Hawkes 1994). At least eight virus resistance genes have been identified in *S. tuberosum* ssp. *andigena*, including PVX resistance genes *Nb* and *Rx1* (Cockerham 1970; Jones 1981). The presence of both *Rx1* and *Nb* in Cara and the fact that *Rx1* is epistatic to *Nb* indicates that there is a lack of selection pressure on the *Nb* gene in this cultivar.

Genetic and molecular analyses have demonstrated that many *R* genes and *R* gene homologues are clustered in the genome of different species (Ellis et al. 1999; Meyers et al. 1998; Parniske et al. 1997). Based on these findings and more detailed analyses of linked *R* genes it has been proposed that the evolution of *R* genes involves intragenic recombination, unequal crossing-over and gene conversion (Ellis et al. 1999; Meyers et al. 1998; Parniske et al. 1997). Resistance to PVX maps to two major clusters of R genes in potato. The cluster located on chromosome V contains at least five resistance loci: *R1* confering resistance to *Phytophthora infestans* (Leonards-Schippers et al. 1992); *Rx2* and *Nb* confering extreme and hypersensitive resistance to PVX (De Jong et al. 1997; Ritter et al. 1991); *Gpa* and *Gpr1* that confer resistance to the potato cyst nematode species *Globodera pallida* and *Globodera rostochiensis* (Kreike et al. 1994; Rouppe van der Voort et al. 1998). The resistance gene cluster located on chromosome XII contains *Rx1* and *Gpa2*, and these gene confer resistance to PVX and *Globodera pallida*, respectively (Bendahmane et al. 1997; Rouppe van der Voort et al. 1997). *Rx1*, *Rx2* and *Gpa2* were characterised at the molecular level: Rx2 shows 95% and 88.7% sequence identity to the Rx1 and

the Gpa2 proteins, respectively. The high homology between sequences from these two clusters indicates that there is some evolutionary relationship between these resistance genes (Bendahmane et al. 2000; Van der Vossen et al. 2000). Interestingly, marker 98L, which is tightly linked to *Nb*, showed significant homology at the nucleotide level to sequences within the R gene cluster on chromosome XII (Van der Vossen et al. 2000) (Fig. 4). The presence of homologous sequences on two different chromosomes in cv. Cara could be explained by unequal crossing-over during recombination of *R* gene clusters or gene conversion between unlinked loci (Bendahmane et al. 1999; Meyers et al. 1998; Parniske et al. 1997).

The cloning and characterisation of the *Nb* gene might reveal new domains involved in the recognition of pathogen elicitors and could provide some clues to explain the differences between HR type of resistance (*Nb*) and extreme resistance (*Rx*) against PVX, which are elicited by two different virus-encoded proteins (Bendahmane et al. 1995; Malcuit et al. 1999). In addition, *Nb* is particularly important because of its genetic linkage to other loci conferring resistance against fungus, nematodes and other viruses. Consequently, the isolation of *Nb* and the physical analysis of this cluster of *R* genes will provide a good experimental system to study the evolution of *R* genes in potato and the mechanisms by which plants generate new recognition specificities.

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