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TMV resistance gene *N* homologues are linked to *Synchytrium endobioticum* resistance in potato

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Abstract The fungus *Synchytrium endobioticum*, the causal agent of potato wart disease, is subject to worldwide quarantine regulations due to the production of persistent resting spores and lack of effective chemical control measures. The selection of *Synchytrium*-resistant potato cultivars may be facilitated by using markers closely linked with a resistance gene or by transferring a cloned gene for resistance into susceptible cultivars. *Sen1*, a gene for resistance to *Synchytrium endobioticum* race 1, was localized on potato chromosome XI in a genomic region which is related to the tobacco genome segment harbouring the *N* gene for resistance to TMV. Using *N* as probe, we isolated homologous cDNA clones from a *Synchytrium*-resistant potato line. The *N*-homologous sequences of potato identified by RFLP mapping a family of resistance gene-like sequences closely linked with the *Sen1* locus. Sequence analysis of two full-length *N*-homologous cDNA clones revealed the presence of structural domains associated with resistance gene function. One clone (Nl-25) encodes a polypeptide of 61 kDa and harbours a Toll-interleukin like region (TIR) and a putative nucleotide binding site (NBS). The other clone (Nl-27) encodes a polypeptide of 95 kDa and harbours besides the TIR and NBS domains five imperfect leucine-rich repeats (LRRs). Both clones have at their amino terminus a conserved stretch of serine residues that was also found in the *N* gene, the *RPP5* gene from *Arabidopsis thaliana* and several other resistance gene homologues, suggesting a function in the resistance response. Cloning of the disease resistance locus based on map position and the establishment of PCR-based marker assays to assist selection of wart resistant potato genotypes are discussed.

Key words Plants · Disease resistance · Host-pathogen interaction · *Synchytrium endobioticum* · Potato

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Introduction

The study of the inheritance of plant resistance in flax and the inheritance of virulence in the rust fungal pathogen *Melampsora linii* led to formulation of the classical gene-for-gene model. This model proposes the requirement of complementary pairs of dominant genes, one in the host and the other one in the pathogen, for resistance to occur (Flor 1971). Over the last 6 years the cloning of plant disease resistance genes has expanded our understanding of host-pathogen interaction at the molecular level. The first plant resistance gene isolated that corresponds to the classic gene-for-gene model was *Pto* from tomato, which confers resistance to races of *Pseudomonas syringae* pv. tomato bacteria carrying the avirulence gene *avrPto* (Martin et al. 1993). The *Pto* gene encodes a serine-threonine protein kinase, suggesting that a phosphorylation signaling cascade is part of the mechanism leading to disease resistance (Loh and Martin 1995; Zhou et al. 1995). *Pto*-mediated resistance requires the presence

of a second gene, *Prf*, which encodes a protein with a leucine zipper, nucleotide-binding site and leucine-rich repeat motifs (Salmeron et al. 1996). These motifs had been found earlier in several other disease resistance genes, with the leucine-rich repeats being a common theme among all.

RPS2 and *RPM1*, two resistance genes from *Arabidopsis thaliana* conferring resistance against *Pseudomonas syringae* bacteria expressing avirulence genes *avrRpt2* (*RPS2*), *avrRPM1* (*RPM1*) or *avrB* (*RPM1*), contain leucine-rich repeats together with a leucine zipper and nucleotide-binding domains (Bent et al. 1994; Grant et al. 1995; Mindrinos et al. 1994). The tobacco mosaic virus (TMV) resistance gene *N* from tobacco, the *L6* and *M* rust resistance genes from flax and the *Arabidopsis RPP5* gene that specifies resistance to the downy mildew pathogen *Peronospora parasitica* all encode proteins with leucine-rich repeats and nucleotide-binding sites combined with an amino-terminal domain similar to that of the cytoplasmic domains of the *Drosophila* TOLL protein and the interleukin-1 receptor (IL-1R) in mammals (TIR-domain, Anderson et al. 1997; Lawrence et al. 1995; Parker et al. 1997; Whitham et al. 1994). The rice *Xa21* and *Xa1* genes which confer resistance to different races of *Xanthomonas oryzae* pv. *oryzae* both carry a leucine-rich repeat motif, while *Xa21* also has a serine-threonine kinase-like domain (Song et al. 1995; Yoshimura et al. 1998). Tomato genes *Cf-2*, *Cf-4* and *Cf-9* conferring resistance to races of the fungus *Cladosporium fulvum* encode putative membrane-anchored glycoproteins with extracellular leucine-rich repeats (Dixon et al. 1996; Jones et al. 1994; Thomas et al. 1997).

In addition to these structural similarities, resistance genes from a wide variety of plant species exhibiting different pathogen specificities also show significant identities in their amino acid sequence (Hammond-Kosack and Jones 1997). The TIR region of the *N* gene, for example, has 42% and 53% amino acid sequence identity with the TIR region of *L6* and *RPP5*, respectively. These genes originate from fairly unrelated plant species such as tobacco, flax and *Arabidopsis*. *Xa21* from rice and *Cf-2* from tomato show 35% amino acid sequence identity in the amino-terminal region of the protein.

These observations are the basis for experimental approaches to isolate new disease resistance genes that are only known by map position and by their specific resistance phenotype. Oligonucleotide primers designed for conserved sequences from coding regions of *N*, *RPS2* and *L6* were used to amplify nine classes of resistance gene analogues from soybean. Several members of these classes map near known resistance genes (Kanazin et al. 1996). In a similar study, eight different classes of resistance gene-like sequences (RGLs) were derived by the polymerase chain reaction (PCR) from potato some of which were correlated by map position with resistance loci within the related tomato and potato genomes (Leister et al. 1996).

In this paper we describe the localization of a potato gene for resistance to *Synchytrium endobioticum* (*Sen1*) in a region of the potato genome that shows synteny with the region of the tobacco genome harbouring the gene *N* for TMV resistance (Leister et al. 1996). The fungus *S. endobioticum*, the causal agent of potato wart disease, is an obligate soil-borne pathogen that produces persistent resting spores (Hampson 1996). A total of ten pathotypes were recently recorded in Germany (Langerfeld et al. 1994). We show that the *Sen1* locus is linked with resistance gene-like sequences that are homologous to the *N* gene. Using the gene *N* from tobacco as a probe, we isolated several homologous cDNA clones from a *Synchytrium*-resistant potato genotype and analysed them for linkage with the *Sen1* resistance locus in potato.

Materials and methods

Plant material

The dihaploid potato line H80.577/1 (line no. 3 in Gebhardt et al. 1989) is resistant to *Synchytrium endobioticum* race 1. This line was crossed as female parent (P3) with the dihaploid line H80.576/16 (line no. 38 in Gebhardt et al. 1989) as male parent (P38). The progeny (population K31) segregated for resistance to *S. endobioticum*. One hundred and eleven lines were evaluated for resistance.

Test for resistance to *S. endobioticum*

Resistance tests were performed with *S. endobioticum* race 1 on tubers by the Pflanzenschutzamt Lübeck (Lübeck, Germany) and the Bayerische Landesanstalt für Bodenkultur und Pflanzenbau (Freising, Germany) based on the methods of Glynne (1925) and Lemmerzahl (1930).

Restriction fragment length polymorphism (RFLP) analysis and linkage mapping

RFLP analysis and linkage mapping was performed as described in Gebhardt et al. (1989). An RFLP linkage map covering all 12 potato chromosomes was constructed in population K31 which is described elsewhere (Schäfer-Pregl et al. 1998). *N*-homologous cDNA clones and PCR-derived resistance gene like sequences 3.3.3 and 3.3.13 (Leister et al. 1996) were used as RFLP marker probes on population K31.

cDNA cloning

polyA(+) mRNA was isolated from leave and stem tissue of the *Synchytrium*-resistant genotype H80.577/1 according to standard procedures (Sambrook et al. 1989). For cDNA synthesis and cloning, the ZAP-cDNA Synthesis and ZAP-cDNA Gigapack II Gold cloning kits were employed (Stratagene). The cDNA was cloned into *Eco*RI/*Xho*I-predigested ZAP Express™ lambda vector (Stratagene). Approximately 4×10^5 primary plaques were plated,

transferred to nitrocellulose filters and hybridized with the *N* gene internal *Bam*HI fragment (Whitham et al. 1994). Low-stringency hybridization conditions were applied for screening the library (Sambrook et al. 1989). Hybridizing phage plaques were carried through several rounds of plating and hybridization in order to obtain a single recombinant clone. The ExAssist helper phage with the *E. coli* XLOLR strain was then used to excise the pBK-CMV phagemids from the ZAP Express vector (Stratagene). Small-scale and large-scale plasmid preparations were performed according to standard techniques using the Qiaprep Spin Plasmid Kit (Quiagen) and the JET Star Plasmid Purification System (Genomed).

Sequencing

The cDNA inserts were sequenced with fluorescence-labelled universal and reverse M13 primers using the AutoRead Sequencing Kit (Pharmacia) and the Automated Laser Fluorescent A.L.F.™ DNA Sequencer from Pharmacia LKB. For sequencing full-length cDNA clones Exonuclease III deletions were generated with the ExoIII/Mung Bean Nuclease Deletion Kit (Stratagene). Each region of the cDNA clones was sequenced at least twice. Sequences were subjected to data bank analysis using the BLAST algorithms (Altschul et al. 1990). Comparative sequence analysis were performed with the BESTFIT and PILEUP programmes of the GCG Wisconsin Sequence Analysis Package.

Results

RFLP mapping of a gene for resistance to *Synchytrium*

Of 111 plants of population K31 analysed, 34 were resistant to *Synchytrium endobioticum* race 1, and 77 were susceptible. If a single dominant resistance allele is assumed to be present in the heterozygous state in parent P3, the expected segregation ratio of 1:1 was distorted towards susceptibility ($\chi^2 = 16.66$, $P < 0.0001$). Linkage analysis with all RFLP alleles segregating in the K31 population (Schäfer-Pregl et al. 1998) revealed linkage with markers *GP125* and *CP58*, both of which are located in a distal segment on linkage group XI of potato (Gebhardt et al. 1991). Both RFLP markers have been mapped in tobacco to the introgressed region of *N. glutinosa*, which harbours the *N* gene for resistance to TMV (B. Baker, unpublished). The linkage map of chromosome XI as derived from meiotic recombination in the resistant parent P3 and the susceptible parent P38 is shown in Fig. 1. The *Sen1* locus occupied a position 5.8 cM distal to *CP58* on linkage group XI of parent P3.

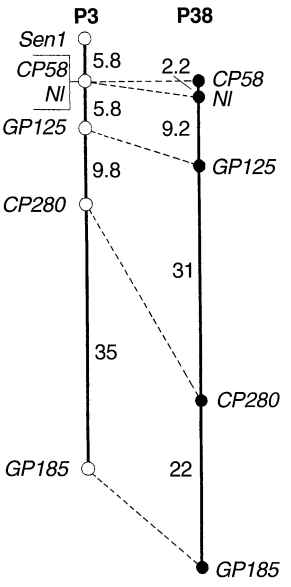


Fig. 1 Map position of the *Sen1* resistance locus on linkage group XI of potato. The *left* linkage group is based on meiotic recombination events in the female parent (*P3*) of population K31, the *right* linkage group is derived from the male parent (*P38*). Genetic distances (in cM according to Kosambi 1944) between loci are indicated. The RFLP loci shown were informative for both parents (connected by *dotted lines*)

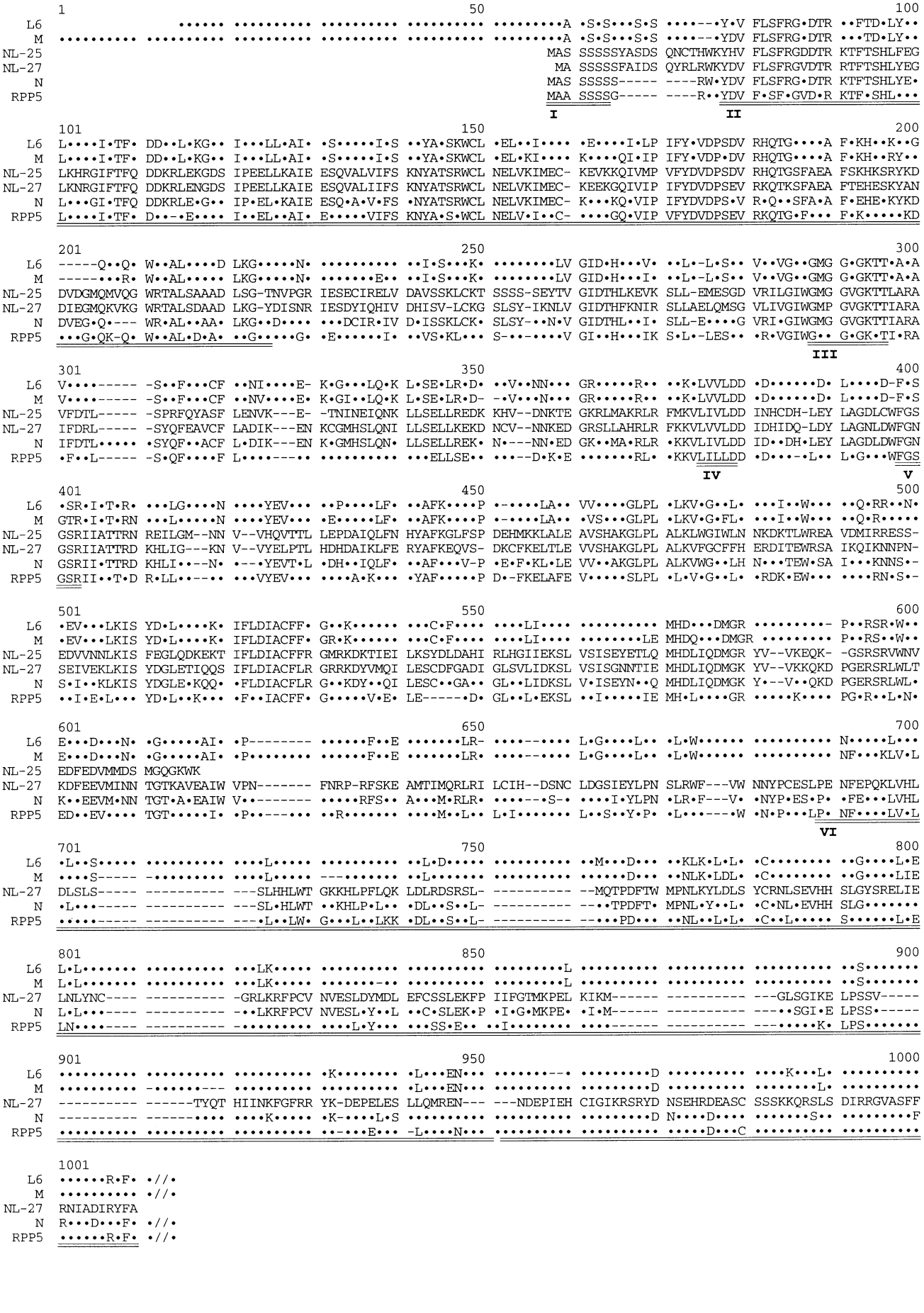
Cloning and sequencing of *N* homologous cDNAs from *Synchytrium*-resistant potato

Using a TMV resistance gene *N* internal *Bam*HI fragment as a probe, we isolated and purified 21 cDNA clones from a cDNA library generated from the *Synchytrium*-resistant parent P3 of population K31 (see Materials and methods). After in vivo excision of the phagemids the 5′ ends of 17 of the 21 clones were sequenced, and the sequence was compared with the databank. Twelve cDNA clones showed homology to the *N* gene, while the remaining 5 genes showed homology to other sequences unrelated to known resistance genes (data not shown). Cloning of genes unrelated to resistance genes was probably due to the low-stringency hybridization conditions employed in the screen (Materials and methods).

Four of the *N*-homologous cDNA clones were sequenced completely. These clones were designated Nl-2, Nl-22, Nl-25 and Nl-27 (N-like). Clones Nl-2 and Nl-22 were both incomplete cDNA clones and consequently omitted from further analysis (data not shown).

Clone Nl-25 appeared to be a full-length cDNA clone. The cDNA is 2063 base pairs (bp) long and encodes a protein of 533 amino acids that shares significant similarities with other disease resistance genes (Fig. 2 and see below). The *N*-homologous reading frame of clone Nl-25 is preceded by 104 bp and followed by 338 bp of untranslated sequence. The cDNA clone terminates in a 22-bp-long polyA tail. The 5′ untranslated region does not show any sequence homology to plant disease resistance genes, while the first 280 bp of the 3′ untranslated region is approximately 74% homologous to the TMV resistance gene *N* (data not shown). When the 338-bp 3′ region is translated, all three translation products have several stop codons, and only one of the three translated sequences that harbours seven stop codons shows some sequence identities with the *N* gene product. The possibility that this clone may represent a differentially spliced gene is discussed below.

With 2919 bp cDNA Nl-27 was the largest clone sequenced. This clone encodes a protein of 821 amino acids that is homologous to NL-25 and other disease


I
II
III
IV
V
VI

resistance genes (Fig. 2 and see below). The *N*-homologous reading frame of clone Nl-27 is preceded 5′ by 161 bp and followed 3′ by 270 bp of untranslated sequence. The cDNA clone terminates in a 25-bp-long polyA tail. The 5′- and 3′-untranslated regions do not show any sequence homologies to other plant disease resistance genes.

Nl-25 and Nl-27 encode disease resistance gene homologues

Clones Nl-25 and Nl-27 translate into proteins with 533 and 821 amino acids, respectively, representing proteins of 60.914 and 94.895 kDa. The amino acid sequence of both proteins is shown in Fig. 2. Both clones belong to the Toll-interkleukin 1 region-harbouring class of disease resistance genes with which they share the highest sequence identities (Hammond-Kosack and Jones, 1997). When compared to other members of this class, both proteins NL-25 and NL-27 have the highest sequence identity to the TMV resistance protein N (76% for NL-25 and 75% for NL-27). Lower sequence identities are found to RPP5 (61% for NL-25 and 58% for NL-27), M (60% for NL-25 and 56% for NL-27), and L6 (60% for NL-25 and 55% for NL-27).

When the two proteins were screened for regions that are implicated in resistance gene function, both were found to contain the Toll-interkleukin 1 region (Region II, Fig. 2). Furthermore, they have the kinase 1a domain or P-loop region also found in the N protein (Region III, Fig. 2; Whitham et al. 1994). Protein NL-27 shows, however, a significant deviation from the consensus sequence in that it contains a proline instead of a glycine at the third position (NL-27, Region III, Fig. 2). The kinase 2 domain is present in both proteins and harbours the invariant aspartate that is believed to coordinate the metal ion binding required for the phospho-transfer reactions (Region IV, Fig. 2; Hammond-Kosack and Jones 1997; Traut 1994). The kinase 3a domain containing an arginine that in other proteins interacts with the purine base of ATP is present in both proteins (Region V, Fig. 2; Hammond-Kosack and Jones 1997; Traut 1994). Among the two proteins, only NL-27 harbours five imperfect leucine-rich repeats (Region VI, Fig. 2).

Interestingly, the amino terminus of the putative NL-25 and NL-27 proteins have a conspicuous stretch of serines that are preceded by the methionine and an alanine. This is also present in the N and the RPP5 protein (Region I, Fig. 2). This motif is also found at the amino terminus of three other N-like proteins from potato (Hehl and Gebhardt, unpublished results) and in several disease resistance gene homologues in the *Arabidopsis* database (data not shown).

Table 1 Comparison of peptides 3.3.3 and 3.3.13 with the corresponding regions within the proteins NL-25 and NL-27

	NL-25[a]	NL-27[a]	RGL 3.3.3[b]	RGL 3.3.13[b]
NL-25[a]	–	58%[d]	52%[d]	57%[d]
NL-27[a]	78%[a]	–	67%[d]	77%[d]
RGL 3.3.3[b]	73%[c]	81%[c]	–	63%[d]
RGL 3.3.13[b]	78%[c]	85%[c]	79%[c]	–

[a] The regions in protein NL-25 and NL-27 comprise amino acids 235–397 and 236–396, respectively
[b] RGL 3.3.3 and RGL 3.3.13 are 150 and 151 amino acids long, respectively
[c] Per cent similarity
[d] Per cent identity

Linkage of *N*-homologous potato cDNAs and other potato RGL sequences to *Sen1*

When used as probes on genomic Southern gel blots of potato, cDNA clones Nl-2, Nl-22, Nl-25 and Nl-27 hybridized to non-identical gene families with multiple copies (not shown). In the mapping population BC916[2] used for RFLP map construction (Gebhardt et al. 1991), all four probes identified an RFLP locus (*Nl*) which cosegregated with marker *CP58* on linkage group XI (not shown). The same locus (*Nl*) was identified on linkage group XI of the K31 map (Fig. 1) when using as probe Nl-25 or Nl-27 cDNA or the PCR-derived RGL sequences 3.3.3 and 3.3.13 (Leister et al. 1996) which share sequence similarity with *N* and cDNA clones Nl-25 and Nl-27 (Table 1). *Nl* was most closely linked with marker *CP58*. The resistance locus *Sen1* mapped distal to *Nl* based on 6 recombinant plants in a total of 103 plants that were scored for both loci (Fig. 1). All 6 recombinant plants were susceptible to *Synchytrium*.

Fig. 2 Amino acid sequence of NL-25 and NL-27 compared to disease resistance proteins L6, M, N, and RPP5. Amino acids in L6, M, N, and RPP5 that are identical to either NL-25 or NL-27 are given in a *single letter code*, while amino acids not conserved are represented by a (•). Gaps are indicated by a (−). The putative functional domains of the deduced proteins are *underlined twice* and represented by *Roman numbers*: *I* a conserved stretch of amino terminal serines, *II* the Toll-Interleukin-1 receptor-similar domain, *III* P-loop or kinase 1a domain, *IV* kinase 2 domain, *V* kinase 3a domain, *VI* leucine-rich repeats. The carboxy terminal amino acids of L6, M, N, and RPP5 are omitted (•//•)

Discussion

A single, dominant gene for resistance to *Synchytrium endobioticum*, *Sen1*, has been located on potato linkage group XI. This region of the potato genome shows

synteny with a region of the tobacco genome which harbours the *N* gene for resistance to tobacco mosaic virus: potato RFLP markers that are linked with *Sen1* crosshybridized with tobacco DNA and detected homologous RFLP loci that were closely linked with *N* (B. Baker, unpublished results). This observation, together with the notion that genes for resistance to different types of pathogens are structurally similar, prompted us to search for candidate sequences for the potato gene *Sen1* by isolating potato homologues of the tobacco *N* gene. Several *N*-homologous potato cDNA clones were isolated, which ultimately identified the locus *Nl* in a distal position on linkage group XI. *Nl* and *Sen1* were separated by 6 susceptible, recombinant plants, however. This result may indicate that the *Sen1* gene has no similarity with *N*-homologous sequences and that the similarity between map positions occurred by chance alone. It cannot be excluded, however, that *Sen1* is a member of a clustered potato resistance gene family which shares a common ancestor with the *N* gene of tobacco. Meiotic instability of the *Sen1* gene instead of recombination events may be the reason for the susceptibility of the 6 recombinant plants separating the *Sen1* and *Nl* loci. Meiotic instability of disease resistance genes has been demonstrated in the *Rp1* region of maize based on flanking RFLP markers (Sudupak et al. 1993). As no RFLP marker flanking *Sen1* on the distal end of linkage group XI was available, the hypothesis of meiotic instability could not be tested in our mapping population. The presence of more than one *N*-related sequence in this region of potato chromosome XI was supported by the observation that several, non-identical RFLPs were detected by *N*-related resistance gene-like sequences Nl-25, Nl-27, 3.3.3 and 3.3.13 in this region (Leister et al. 1996; this paper). Moreover, genes for resistance to potato viruses Y (PVY) and A (PVA) are located in the same region (Hämäläinen et al. 1997, 1998; Brigneti et al. 1997). Resistance gene-like sequences that were highly homologous (>90 percent identity) to markers 3.3.3 and 3.3.13 used in this study cosegregated with the PVY resistance locus Ry_{adg}, whereas the PVA resistance locus Ra_{adg} occupied a map position 6.8 cM distal to Ry_{adg} (Hämäläinen et al. 1998). Based on mapping experiments carried out in unrelated potato germplasm, *Nl* and *Sen1* occupy, therefore, similar positions as Ry_{adg} and Ra_{adg}, respectively.

The genes corresponding to full-length cDNA clones Nl-25 and Nl-27 may or may not be located at the *Nl* locus on linkage group XI. A number of genomic restriction fragments which did not segregate in the mapping populations tested and could, therefore, not be mapped were detected by the cDNA sequences. These unmapped genomic fragments may correspond to additional loci unlinked with *Nl* on linkage group XI.

The deduced polypeptides NL-25 and NL-27 have as yet unknown functions. The fact that the polypeptides have a structure similar to other disease resistance gene products suggests that they function as resistance determinants of yet unknown specificities or as susceptibility determinants. They may also be required for other functions. The sequence similarity of the amino terminus of N to the cytoplasmic domains of the *Drosophila* Toll and the human interleukin-1 receptor protein led to a model that N might trigger an intracellular signal transduction cascade similar to Toll and IL-1R (Whitham et al. 1994). Both of the *N*-like putative proteins of potato reported here have high homology in the Toll-interleukin-1R domain, which suggests that both proteins may be involved in similar signal transduction pathways. A putative nucleotide-binding site domain is also present in both deduced polypeptides NL-25 and NL-27, although a proline is present instead of a glycine in the P-loop region of NL-27 that may interfere with nucleotide-binding. The lack of a leucine-rich repeat region in NL-25 could either be an intrinsic property of the functional protein or originate from differential splicing. There are truncated versions of both L6 and N that lead to proteins with fewer leucine-rich repeats (Lawrence et al. 1995; Whitham et al. 1994). The deduced protein NL-25 has 313 amino acids at its carboxy terminus that are identical to those found in the amino terminal sequence of the polypeptide deduced from the partial cDNA NL-22. Furthermore, NL-22 has 263 additional carboxy terminal amino acids with leucine-rich repeats (data not shown). Both clones possibly originate from the same gene and may represent differentially spliced products. NL-25 may thus be a truncated version of a larger protein.

The conspicuous stretch of serine residues at the amino terminal end of the NL-25, NL-27, N and RPP5 proteins as well as on other disease-resistant gene homologues suggests a functional role of these amino acids. It is most tempting to speculate that these serines may serve as substrates for phosphorylation (Pawson and Scott 1997). There are no examples of similar stretches of serines with functional significance at the amino terminal end of other known proteins. However, there are cases of serine phosphorylation in the amino terminal region of proteins that are relevant to protein function. An example of amino terminal serines required for gene function is the *Drosophila* CACTUS protein. Regulated proteolysis of CACTUS, the cytoplasmic inhibitor of the Rel-related transcription factor Dorsal, is an essential step in patterning of the *Drosophila* embryo. CACTUS stability is regulated by amino-terminal serine residues necessary for signal responsiveness as well as by a carboxy-terminal PEST domain (Liu et al. 1997). The nuclear respiratory factor 1 (NRF-1) is an example where phosphorylated serines are clustered. NRF-1 is a transcriptional activator that acts on a diverse set of nuclear genes required for mitochondrial respiratory function in mammalian cells. Phosphorylation occurs on serine residues within a concise amino terminal domain with the major sites of

phosphate incorporation at serines 39, 44, 46, 47, and 52 (Gugneja and Scarpulla 1997). It will be interesting to learn if the conserved cluster of serines in some of the disease resistance genes is required for gene function.

Potato wart is a quarantine disease. Marker-assisted selection for resistance at an early stage of cultivar development may be useful in potato breeding programmes. Knowing the position of the resistance locus *Sen1* on the molecular map of potato provides access to a variety of DNA-based markers (Gebhardt et al. 1991; Leister et al. 1996; Hämäläinen et al. 1997, 1998; Brigneti et al. 1997) for which PCR-based marker assays can be developed for specific potato germplasm carrying resistance alleles at the *Sen1* locus.

The identified linked molecular markers can also be used for map-based cloning approaches towards isolation of the *Synchytrium* resistance gene. Map-based cloning was employed for isolation of the *Pto* gene from tomato, *RPS2*, *RPM1* and *RPP5* from *Arabidopsis* and the *Hs1* gene from sugar beet which confers resistance to the beet cyst nematode *Heterodera schachtii* (Bent et al. 1994; Cai et al. 1997; Grant et al. 1995; Martin et al. 1993; Mindrinos et al. 1994; Parker et al. 1997). The markers linked to *Synchytrium* resistance may serve as a basis for fine mapping of the resistance locus similar to the identification of linkage between *Cf-2*/*Cf-5* and the *Mi* resistance loci in tomato which confers resistance to root-knot nematodes (Dickinson et al. 1993).

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