Molecular cloning of the potato *Gro1-4* gene conferring resistance to pathotype Ro1 of the root cyst nematode *Globodera rostochiensis*, based on a candidate gene approach

Jürgen Pael1,*, Heike Henselewski1, Jost Muth1,*, Khalid Meksem2, Cristina M. Menéndez1,*, Francesco Salamini1, Agim Ballvora1 and Christiane Gebhardt1,2

1MPI für Züchtungsforschung, Carl von Linne Weg 10, 50829 Köln, Germany, and
2Department of Plant, Soil and General Agriculture, Southern Illinois University at Carbondale, Carbondale, IL 62901-4415, USA

Received 10 November 2003; revised 14 January 2004; accepted 19 January 2004.

*For correspondence (fax +49 221 5062 413; e-mail gebhardt@mpiz-koeln.mpg.de).
*Current address: ALTANA Pharma AG, Byk-Gulden-Str.2, 78467 Konstanz, Germany.
*Current address: Fraunhofer IME, Worlingerweg 1, 52074 Aachen, Germany.
*Current address: Universidad de la Rioja, Departamento de Agricultura y Alimentación, C/ Madre de Dios 51, 26006 Logroño, Spain.

Summary

The endoparasitic root cyst nematode *Globodera rostochiensis* causes considerable damage in potato cultivation. In the past, major genes for nematode resistance have been introgressed from related potato species into cultivars. Elucidating the molecular basis of resistance will contribute to the understanding of nematode–plant interactions and assist in breeding nematode-resistant cultivars. The *Gro1* resistance locus to *G. rostochiensis* on potato chromosome VII co-localized with a resistance-gene-like (RGL) DNA marker. This marker was used to isolate from genomic libraries 15 members of a closely related candidate gene family. Analysis of inheritance, linkage mapping, and sequencing reduced the number of candidate genes to three. Complementation analysis by stable potato transformation showed that the gene *Gro1-4* conferred resistance to *G. rostochiensis* pathotype Ro1. *Gro1-4* encodes a protein of 1136 amino acids that contains Toll-interleukin 1 receptor (TIR), nucleotide-binding (NB), leucine-rich repeat (LRR) homology domains and a C-terminal domain with unknown function. The deduced Gro1-4 protein differed by 29 amino acid changes from susceptible members of the *Gro1* gene family. Sequence characterization of 13 members of the *Gro1* gene family revealed putative regulatory elements and a variable microsatellite in the promoter region, insertion of a retrotransposon-like element in the first intron, and a stop codon in the NB coding region of some genes. Sequence analysis of RT-PCR products showed that *Gro1-4* is expressed, among other members of the family including putative pseudogenes, in non-infected roots of nematode-resistant plants. RT-PCR also demonstrated that members of the *Gro1* gene family are expressed in most potato tissues.

Keywords: *Solanum tuberosum, Globodera rostochiensis*, nematodes, resistance gene, candidate gene family.

Introduction

About 60 parasitic nematode species feed on potato plants (Jensen et al., 1979). Species causing yield reduction are cyst nematodes of genus *Globodera*, root lesion nematodes of genus *Pratylenchus*, and root knot nematodes of genus *Meloidogyne* (Brodie, 1999). *Globodera rostochiensis*, the golden cyst nematode, and *G. pallida*, the white cyst nematode, are considered the most damaging for potato production (Brodie, 1984; Evans and Trudgill, 1992). Both *Globodera* species are distributed worldwide and occur either intermixed or individually in potato production areas (Mai, 1977). They presumably originated in the Andean mountains of South America where they co-evolved with their hosts (Evans and Brodie, 1980).

*Globodera* root cyst nematodes have as hosts Solanaceae species, among those important crops such as potato (*Solanum tuberosum*), tomato (*Lycopersicon esculentum*),
and eggplant (*S. melongena*; Southey, 1965). In contrast to other plant parasitic nematodes (e.g. *Heterodera schachtii*), propagation of *Globodera* on the model plant *Arabidopsis thaliana* is not possible (Sijmons et al., 1991). *Globodera* larvae rest in the soil in eggs encapsulated in a small (diameter < 1 mm), spherical cyst. In spring and early summer, excretions of potato roots stimulate the hatching of the larvae and act as an attractant. Mechanically supported by their stylet and enzymatically by oesophageal secretions, the nematodes invade the potato roots. In the inner cortex, only the females induce the formation of a large syncytium feeding site, whereas the males die after mating. The syncytium expands until it is in close proximity to the xylem. The female produces 200–300 eggs, and its spherically enlarged body penetrates the root. The animal’s body passes through a white and yellow (only *G. rostochiensis*) colored stage before it develops into a brown cyst containing infectious larvae and dies. The cysts are remarkably resistant to unfavorable environmental conditions and nematicides and can persist more than 10 years in the soil, which makes control difficult (reviewed in Sijmons, 1993; Williamson and Hussey, 1996).

Genes for nematode resistance have been introgressed in potato cultivars from other tuber bearing *Solanum* species such as *S. andigena*, *S. vernei*, and *S. spegazzinii* (Ross, 1962, 1966). Several of these genes have been mapped, including genes for resistance to *G. rostochiensis*, *G. pallida*, and *Meloidogyne chitwoodi* (reviewed in Gebhardt and Valkonen, 2001). *Gro1*, a major dominant locus conferring resistance to all pathotypes of *G. rostochiensis* has been localized on potato chromosome VII (Barone et al., 1990), and high-resolution mapping assigned Gro1 to an interval of 1.4 cM (Ballvora et al., 1995). Short sequence signatures conserved between the tobacco *N* gene for resistance to tobacco mosaic virus (Whitham et al., 1994) and *RPS2* of *A. thaliana* conferring resistance to the bacterium *Pseudomonas syringae* (Bent et al., 1994; Mindrinos et al., 1994) were used to amplify from the potato genome several different resistance-gene-like (RGL) DNA fragments. The RGL fragments *St*332 and *St*334 co-localized with Gro1 and identified a clustered family of closely related genes at the Gro1 locus (Leister et al., 1996). *St*332 and *St*334 were 95% identical at the nucleotide level and shared 72% sequence similarity with the nucleotide-binding (NB) domain of the *N* gene from tobacco.

The cloning of four nematode resistance (*R*) genes from plants has been reported. *Hs*1pro-1 from sugar beet confers resistance to the beet cyst nematode *H. schachtii* (Cai et al., 1997). The tomato gene *Mi-1.2* on chromosome 6 mediates resistance to three different root knot nematode species of the genus *Meloidogyne* and to the potato aphid *Macrosiphum euphorbii* (Milligan et al., 1998). The *Gpa2* gene on potato chromosome XIX controls pathotype-specific resistance to *G. pallida* (Van der Vossen et al., 2000). Recently, the tomato gene *Hero* on chromosome 4 was cloned, which confers resistance to various pathotypes of *G. rostochiensis* and *G. pallida* (Ernst et al., 2002). Five major classes of plant genes for resistance to different types of pathogens are currently described (reviewed in Dangl and Jones, 2001). All nematode *R* genes, except *Hs*1pro-1, are structurally related and fall into the class of *R* genes that share a leucine zipper (LZ) motif, an NB, and a leucine-rich repeat (LRR) domain. *Hs*1pro-1 codes for an unusual protein with an LRR domain, which does not fit a common consensus motive for plant disease *R* genes.

Here, we report the cloning and characterization of the *Gro1* gene family on potato chromosome VII. We show by complementation analysis that *Gro1-4*, one member of the family, encodes a functional nematode *R* gene. Our cloning strategy relied on the hypothesis that at least one member of the candidate gene family detected by RGL fragments *St*332 and *St*334 at the *Gro1* locus was indeed a gene for resistance to *G. rostochiensis*.

Results

Identification, cloning, and characterization of *Gro1* candidate genes

Six potato genotypes were subjected to Southern gel blot analysis using *TaqI* as restriction enzyme and the Nb-homologous sequence *St*332 as probe (Figure 1). The diploid clones P40 and P18 were the resistant (*Gro1/gro1*) and susceptible (*gro1/gro1*) parent, respectively, of population F1840 used for high-resolution mapping of *Gro1* and *St*332 (Ballvora et al., 1995; Leister et al., 1996). Plant R458 was resistant to *G. rostochiensis* pathotype Ro1 (*Gro1/gro1*) and was one of 121 recombinant plants selected from 1100 plants of population F1840 for having a crossing-over between two markers that flanked the *Gro1* locus (Ballvora et al., 1995). The diploid genotype P6/210 was a progeny of the cross between the diploid clones P41 and P40 and was selected for having both the *Gro1* nematode *R* gene and the *R1* gene for resistance to late blight (genotype *Gro1/gro1*, *R1/r1*; Ballvora et al., 2002). P6/210 was the source of the lambda and BAC genomic libraries. The nematode-susceptible, tetraploid variety Désirée was used for molecular complementation analysis. Three accessions of the diploid, wild potato species *S. spegazzinii*, the historical source of the *Gro1* resistance (Barone et al., 1990; Ross, 1986), were included in the Southern gel blot. The *St*332 probe detected, for each genotype, a distinct *TaqI* fingerprint with 7–16 restriction fragments between 500 and 2000 bp in length (Figure 1). Twelve *TaqI* fragments were shared between the *S. spegazzinii* accessions and resistant as well as susceptible *S. tuberosum* genotypes, indicating similarity between the two species for this particular chromosome.

fragment, but not pointing to any specific \( \text{TaqI} \) candidate gene. The \( \text{TaqI} \) fragments present in the parental genotypes P40, P18, and P41 and inherited by progeny P6/210 and/or R458 were numbered from 1 to 16 (Figure 1). The members of the \( S_{32} \) gene family having these \( \text{TaqI} \) fragments were named, accordingly, from gene 1 to 16. Genes 1, 7, 9, 12, 12b, 13, and 15 were absent in P40, the source of the \( \text{Gro1} \) nematode resistance, and were therefore excluded as \( \text{Gro1} \) candidates. Genes 2–6, 8b, 8, 10, 11, 14, and 16 were present in P40. The nematode-resistant plant R458 lacked, however, genes 3, 6, 8, and 10 (Figure 1), which restricted the \( \text{Gro1} \) candidates to genes 2, 4, 5, 8b, 11, 14, and 16. These seven members of the \( S_{32} \) gene family were present in P40 and inherited by R458 (Figure 1). Genes 2, 4, 5, and 14 cosegregated with nematode resistance in the F1840 population (Leister et al., 1996), whereas gene 8b was linked in coupling phase to the susceptible allele \( \text{gro1} \) of P40 (not shown), excluding this gene as a candidate. The presence of gene 8b in the nematode-resistant plant R458 indicated, however, that the recombination break point in plant R458 was located within the \( S_{32} \) gene cluster linked to \( \text{Gro1} \). Gene 11 mapped to the \( \text{Gro1} \) locus but was also present in the susceptible parent P18, whereas gene 16 did not segregate in population F1840 (Leister et al., 1996).

Lambda and bacterial artificial chromosome (BAC) genomic libraries were screened using \( S_{32} \) as probe. Positive clones were isolated and subjected to fingerprint analysis with \( \text{TaqI} \) restriction enzyme (not shown). Based on presence of individual \( \text{TaqI} \) restriction fragments, BAC and/or lambda clones for all members of the \( S_{32} \) gene family of genotype P6/210 were identified, with the exception of gene 9 (Figure 1; Table 1). Five BACs contained two, and a sixth BAC contained three of the \( \text{Gro1} \) candidate genes. This showed that genes 4 and 14, and 12, 5, and 11 were physically tightly linked. The combination of genes 2, 5, and 11 present in three BACs was used to deduce their linear order 2-11 on BAC clone BA98P9 (Table 1). Whether the genomic clones originated from either P40 or P41 could be inferred from the presence of the corresponding \( \text{TaqI} \) fragments in either P40 or P41 (Table 1; Figure 1), with the

\[
\text{Table 1 BAC and lambda P6/210 genomic clones containing members of the } S_{32} \text{ gene family and their parental origin}
\]

<table>
<thead>
<tr>
<th>BAC clones</th>
<th>Lambda clones</th>
<th>Origin</th>
</tr>
</thead>
<tbody>
<tr>
<td>BA107K18</td>
<td>79, 94</td>
<td>P41</td>
</tr>
<tr>
<td></td>
<td>74, 48</td>
<td>P40</td>
</tr>
<tr>
<td>BA260K12, BA60B11</td>
<td>–</td>
<td>P40</td>
</tr>
<tr>
<td>BA261J14, BA78o5</td>
<td>–</td>
<td>P40</td>
</tr>
<tr>
<td>BA34L19, BA81L19</td>
<td>110</td>
<td>P41</td>
</tr>
<tr>
<td>BA60L16, BA73N5</td>
<td>–</td>
<td>P40</td>
</tr>
<tr>
<td>BA26N18</td>
<td>–</td>
<td>P40</td>
</tr>
<tr>
<td></td>
<td>65, 113</td>
<td>P40</td>
</tr>
<tr>
<td>BA115B4, BA49J20</td>
<td>–</td>
<td>P40</td>
</tr>
<tr>
<td>BA34L19, BA53o21</td>
<td>–</td>
<td>P41</td>
</tr>
<tr>
<td>BA42C17, BA71o17, BA78M21, BA119F19</td>
<td>36</td>
<td>P40</td>
</tr>
<tr>
<td>BA107K18</td>
<td>107</td>
<td>P41</td>
</tr>
<tr>
<td></td>
<td>75</td>
<td>P40</td>
</tr>
<tr>
<td></td>
<td>101</td>
<td>P41</td>
</tr>
<tr>
<td></td>
<td>33</td>
<td>P41</td>
</tr>
<tr>
<td>BA42o6, BA87D4, BA254K3</td>
<td>50</td>
<td>P41</td>
</tr>
<tr>
<td>BA25J5</td>
<td>98</td>
<td>P40</td>
</tr>
<tr>
<td>BA22K21, BA248H17</td>
<td>16</td>
<td>P41</td>
</tr>
<tr>
<td>BA106C14</td>
<td>–</td>
<td>P40</td>
</tr>
</tbody>
</table>

Table 2  PCR-based markers for mapping gene 16 and for assignment of genomic clones either to parent P40 or P41

<table>
<thead>
<tr>
<th>Genomic clone used as marker source</th>
<th>Gene no.</th>
<th>Position of primer pairs</th>
<th>Type of polymorphism</th>
<th>PCR product size (bp)</th>
<th>Primer sequences (5′ → 3′)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BA106C14</td>
<td>16</td>
<td>At T3 end</td>
<td>CAPS (Alu)</td>
<td>629</td>
<td>GACAAAACTGAAATTATAGTACCTTAC</td>
</tr>
<tr>
<td>BA106C14</td>
<td>16</td>
<td>At T7 end</td>
<td>Specific PCR for P40 allele</td>
<td>1713</td>
<td>CTCTCCGTCCTCCTCCGCTGCA</td>
</tr>
<tr>
<td>BA25J5</td>
<td>14</td>
<td>At T7 end</td>
<td>SNP</td>
<td>1684</td>
<td>GCTACGATTCAGTGACTACGACGAC</td>
</tr>
<tr>
<td>Lambda 10</td>
<td>3</td>
<td>At T3 end</td>
<td>Specific PCR for P40 allele</td>
<td>2089</td>
<td>GGCTACAGTGTCTTCTAGGA</td>
</tr>
<tr>
<td>Lambda 110</td>
<td>3</td>
<td>At T7 end</td>
<td>Specific PCR for P41 allele</td>
<td>487</td>
<td>GGATACTACTGGAGAAACC</td>
</tr>
<tr>
<td>Lambda 107</td>
<td>10</td>
<td>In gene 10</td>
<td>Specific PCR for P41 allele</td>
<td>465</td>
<td>TGCAAACTAAAGGACGCTC</td>
</tr>
<tr>
<td>Subclone b32b of BA98P9</td>
<td>2</td>
<td>In gene 2</td>
<td>Specific PCR for P40 allele</td>
<td>481</td>
<td>ACAACGTTCTAAATCTGTCG</td>
</tr>
<tr>
<td>Lambda 75</td>
<td>11</td>
<td>In gene 11</td>
<td>Specific PCR for P40 allele</td>
<td>449</td>
<td>ATGATTGAACACTGTGGTGGT</td>
</tr>
<tr>
<td>Lambda 101</td>
<td>11</td>
<td>In gene 11</td>
<td>Specific PCR for P41 allele</td>
<td>572</td>
<td>AGATGTGCACTTACCGAAATAT</td>
</tr>
</tbody>
</table>

exception of genes 2–4, 10, 11, 14, and 16. The TaqI fragments characteristic for these genes were, in fact, present in both parents of P6/210 (Figure 1). PCR-based markers were developed, therefore, for mapping gene 16 and for identifying genomic clones having the P40 allele of these genes (Table 2). The PCR-based markers for both ends of BAC BA106C14 including gene 16 mapped in population F1840 to the short arm of chromosome IV, 5 cM proximal to RFLP marker GP180 (not shown), therefore excluding gene 16 as candidate for Gro1, which is located on chromosome VII. Allele-specific markers for genes 2, 3, 10, 11, and 14 identified BAC and lambda genomic clones originating from P40 or P41. The parental origin of genomic clones having gene 4 was deduced from its linkage to gene 14 (Table 1). Based on the assignment of P6/210 TaqI fragments and genomic clones to either the P40 or P41 allele, the St322 gene cluster of the P40 allele linked to Gro1 consisted of at least eight genes (2–6, 8, 11, and 14), whereas the P41 allele linked to gro1 consisted of at least nine genes (1, 3, 7, 9–13, and 15).

Members of the St322 gene family were sequenced from lambda or BAC genomic clones by primer walking, starting with outward-directed primers binding to the St332 homologous region (Accessions AY196151–AY196163). The organization in four exons and three introns of eight genes of the P40 allele linked to Gro1 was determined by sequence comparison to 5′ and 3′ rapid amplification of cDNA ends (RACE) products (see below) and is shown in Figure 2. Database homology searches revealed that the St322 gene family was homologous to the Toll-interleukin 1 receptor (TIR)/NB/LRR class of plant genes for pathogen resistance. The three domains were encoded by the first three exons.

The TIR coding exon I was identified in six of the eight genes and was separated from the NB coding exon II by an intron of variable size because of the insertion of retroelements in intron I of genes 2, 4, and 11. The retroelement insertion in intron I of gene 2 was itself interrupted by another insertion (not shown in Figure 2), resulting in an intron size of 11.2 kbp. In the NB coding region, stop codons at different positions were identified in genes 3, 6, 8, 11, and 14 and were confirmed by sequencing both DNA strands. In addition, the NB coding exon of gene 14 was truncated at the 5′ end. In all eight genes, the NB coding exon was separated from the LRR coding exon III by a small intron having similar size in the different genes. The LRR coding exon of gene 14 contained an inverted repeat element. Exon IV did not show homology to other genes with known function. Based on sequence analysis, genes 2, 4, and 5 encoded putative functional TIR/NB/LRR-type proteins whereas genes 3, 6, 8, 11, and 14 were considered nonfunctional because of internal stop codons. Genes 11 and 14 were excluded therefore as candidates, leaving genes 2, 4, and 5, all linked to the Gro1 allele of P40, as the three remaining candidates for the Gro1 nematode R gene.

Subcloning and complementation analysis

The candidate genes 2, 4, and 5 were subcloned into the binary plant transformation vector pCLD04541 and transferred to the susceptible potato cv. Désirée via Agrobacterium tumefaciens-mediated transformation. The 22-kbp subclone b32b containing gene 2 (Accession AY196153) was obtained from BAC clone BA98P9 (Table 1). Gene 4 (subclone b07c with 14.5 kbp, Accession AY196151)
Figure 2. Structural alignment of eight members of the GroT family linked in coupling phase to the resistant GroT allele of P40 (Accession numbers AY196151–AY196163).

Asterisk (*) indicates stop codon mutations. Triangles indicate retroelement insertions in intron I of genes 2, 4, and 11, and an inverted repeat element insertion in the LRR domain of gene 14. Question mark indicates that no TIR coding exon I was found in the sequenced genomic region. A deletion in genes 4 and 5 is indicated with a ‘roof’ symbol. TIR, Toll/Interleukin-1 receptor homology region; NB, nucleotide-binding domain; LRR, leucine-rich repeat domain. Exons are shown as dark gray boxes. Boxes in light gray are 5’ and 3’ untranslated regions upstream of exon I and downstream of exon IV, respectively.

and gene 5 (subclone b16j with 10.5 kbp, Accession AY196152) were subcloned from BACs BA26N18 and BA60B11, respectively. Subclones b32b, b07c, and b16j included 2.3-, 4.0-, and 2.7-kbp sequence upstream of the putative transcription start site and 3.4-, 1.1- and 3.0-kbp sequence, respectively, downstream of the putative stop site. Between 30 and 37 independent transgenic lines were regenerated per construct and tested for resistance to *G. rostochiensis* pathotype Ro1 (Table 3). All plants transformed with gene 2 or 5 constructs were susceptible. Of 30 lines transformed with the gene 4 construct, 14 lines were resistant and 16 lines were susceptible (Table 3). Candidate gene 4 was therefore able to confer resistance to *G. rostochiensis* pathotype Ro1 and was named *Gro1-4*. Using the b07c construct, the frequency of nematode-resistant transgenic plants was 47%. Five resistant and five susceptible *Gro1-4* transgenic lines were analyzed for the presence of *TaqI* fragment 4 that was absent in untransformed cv. Désirée (Figures 1 and 3). The *TaqI* fragment 4 was present, as expected, not only in the five resistant plants but also in the five susceptible plants tested (Figure 3). It was concluded that the susceptible phenotype of these five plants may be the consequence of position effects, gene silencing, or rearrangements of the transgene.

Structure of the *Gro1-4* nematode resistance gene

The 14.5-kbp sequence of subclone b07c carrying the nematode *R* gene *Gro1-4* was determined (Accession AY196151). No other open-reading frames besides the TIR/NB/LRR gene were found. Intron I included a retroelement and was 5465-bp long. Introns II and III were 76 and 115 bp long, respectively. The putative promoter region contained the ethylene-responsive element ‘AWTTCAAA’ (Montgomery *et al.*, 1993), four CAAT boxes, of which two were in reverse orientation, and the motif ‘CAANNN-NATC’ that is conserved in 5’-upstream regions of circadian clock-controlled light harvesting complex (Lhc) genes (Piechulla *et al.*, 1998). The putative TATA box was part
of a TA microsatellite with variable length in different members of the gene family (Figure 4a). The microsatellite in genes gro1-5 and gro1-6 was shorter when compared to genes gro1-2 and Gro1-4. The microsatellite, and thus a putative TATA box, was missing in genes gro1-3 and gro1-11. Two ATG translation start codons were present in the R gene Gro1-4. Translation from the first ATG results in the formation of a signal peptide (Figure 4b), as predicted by SIGNALP. Based on a purine present at position –3 and +4 (Kozak, 1991), translation more likely starts at the second ATG codon. The deduced amino acid sequence (1136 amino acids, Figure 4c) comprised a TIR homology domain as predicted by PROFILESCAN, an NB domain including the kinase 1a (P-loop), kinase 2, and kinase 3a motifs (Sarasate et al., 1990; Walker et al., 1982), the GLPLAL amino acid signature, and an LRR domain with 11 LRR modules (predicted by PROFILESCAN). The consensus motif of the

LRR modules XLXXLXX[C(N/T)X]LXXXP indicated that the Gro1-4 protein may be located in the cytoplasm (Jones and Jones, 1997). No function could be predicted for the C-terminal part of the resistance protein encoded by exon IV. At the amino acid level, Gro1-4 and gro1-2 were 95% identical to each other and both genes were 90% identical to gro1-5. The deduced Gro1-4 protein differed by 29 amino acids from gro1-2 and gro1-5 (Figure 4c). Sixteen amino acid changes were non-conservative. When compared with functionally characterized plant R genes in the database, Gro1-4 was most closely related to the tobacco N protein for resistance to tobacco mosaic virus (Whitham et al., 1994) and to the RPP1-WSA protein for resistance to Peronospora parasitica from A. thaliana (Botella et al., 1998) sharing 38 and 35% sequence identity, respectively. Homology with the functionally most similar tomato nematode resistance protein Hero (Ernst et al., 2002) was low (24% identity). At the nucleotide level, the NB region of Gro1-4 shared 93 and 92% sequence identity with the tomato NB-LRR R gene homologs Q2 and Q112, which map to syntenic positions on tomato chromosome 7 (Pan et al., 2000).

Expression of the Gro1 gene family

Total RNA was isolated from roots of the nematode-resistant line P6/210 20 days after infection with G. rostochiensis pathotype Ro1. No expression of the Gro1 gene family was detectable on Northern gel blots probed with St322. On a virtual Northern blot of the same tissue, using as template PCR-amplified whole cDNA instead of RNA, the St322 probe detected three cDNA bands of 3.6, 2.3, and 1.8 kb (not shown). The size of the largest 3.6-kbp product corresponded to transcripts predicted for the genes gro1-2, Gro1-4, and/or gro1-5. The smaller cDNA fragments can be products of truncated or alternatively spliced transcripts or of transcripts from unknown members of the Gro1 gene family.

RACE PCR was performed on poly(A) + RNA from roots of P6/210 20 days after infection with the nematode. 5′ and 3′ RACE products of variable length were cloned and sequenced. The in silico assembly of the sequence of the longest 5′ and 3′ RACE products resulted in a putative transcript of 3.6 kb, the same size as detected on the virtual Northern blot (not shown). The deduced amino acid sequence of the 3.6-kbp assembled RACE transcript was
Molecular cloning of the Gro1-4 nematode resistance gene

(a)

<table>
<thead>
<tr>
<th>Gro1-4</th>
<th>1</th>
</tr>
</thead>
<tbody>
<tr>
<td>gro1-2</td>
<td>6</td>
</tr>
<tr>
<td>gro1-3</td>
<td>6</td>
</tr>
<tr>
<td>gro1-4</td>
<td>7</td>
</tr>
<tr>
<td>gro1-5</td>
<td>6</td>
</tr>
<tr>
<td>gro1-6</td>
<td>8</td>
</tr>
<tr>
<td>gro1-11</td>
<td>10</td>
</tr>
</tbody>
</table>

(b)

MQAVNIITNFLKLVLSSCRFVIVK
putative signal peptide

(c)

1 MNQSSLLPSDEIIYDSYVFLSFLRIGEDVRKTVFVDLSLALLEQKCIINTKFDEKLEKGFISPFLVSSIK
71 KRSLALIFKSKYANSTWCLDELTKIMECKNVKQIVPPVPSVDFSTIKSGFEGAESKHEARPQED
141 KVQKARALEEEANISGWLINDTAUHHEARNMEKIAEDIMALGSRHASARNLVMGHSMHVKYKMLG
211 FGSSGVFLIGILGMSGVQKTLRVLVYDNLIRSQFQGACFLHEVDRSDSAQAKGLERLQIEILLSEILVVKLR
Kinase 1a, P-loop motif
281 INDSFEGARMQKQRLOYKVLVLLDDVDHQIDLMLNLAGERSEFGQDSSPIIITTFKDKHLLVKYETEIKYRM
Kinase 2
351 KTLNNYEQLFQHAFKKNFTEFKEFDLSAQVKHTGGLPLALKVGLYGLGRALDWEISVERLQIP
421 ENELLKELEQSGTFGLNTEQKIFLZACFSSGKKEKSRTRILESHPFCSVIGIKMLFRMLLTTLGGRIT
491 IHQILQDMMGHWTVREATTDDPNRSRMWRRDLICPVLELNGLTDKNEGSMHLHTHEEENVFGKAFMQMT
561 RLRLKFLFRNAYVCQGEPFLDELRWLDWHYPSKSLMPFSGQDLNLKMLKRSRRIQLWKTSDLGKLKY
631 MNLSHSSQKLIRTPDFSTMVNLRLVERCSLTVNESIFNLGKVLNLLNKLSRNLTKNFLRRKLEL
701 LVLTCGLKLRTFSISBEHNNLCAELYLGLATSLSLSPASVENLSGVVINLICTYHELESPLSSFLRKLCLK
LRR (leucine-rich repeat) - region
771 TDLVSGCSKLLKPNLDDLGLVGLELLHCTHTAIATIYPSMSSLKNNLKLHSLSGCNALSSQVSSHSGQKS
841 MGVTNPQNSGCLSLMTMLDSCNSISGIGTINLNGFLSSVZILLNNGNSHAPAISRFPRLRRKCLH
910 CCGRLSPEPFLPSKIGFANECSTSLMSIDQTVYMLSDATFRNRCQVLVENKQHTSMVDSLLEQLMLEAL
980 YMDRFLYVMEIPSWFTYKSWTGKSQKSVALTIFIWFPTGFTFCVILDEDEELILFRLGRTEHKYWGL
1050 ENNPLNLKRYDLQKINSTFSFPSSKPGGGLDFLITIAEQERSWKLENDLDYYRNNAFQLEFSACDCH
1120 YQVMTVGGILGRVLVSYN*
most closely related to the Gro1-4 deduced nematode resistance protein, differing by eight amino acids (not shown).

To dissect the expression of individual members of the Gro1 gene family, RT-PCR experiments were performed with two primers spanning intron II (Figure 2), which bind to absolutely conserved regions in all sequenced members of the Gro1 family. A 500-bp cDNA product was generated from total RNA of uninfected roots of P6/210 and cloned. The nucleotide sequences of 21 individual clones were compared to 13 highly homologous Gro1 genomic sequences originating from P41 or P40 alleles, including Gro1-4. Between one and six cDNA products were identified that corresponded to 7 of 13 Gro1 genomic sequences and included both P40 and P41 alleles (Figure 5). Among those were two products that corresponded to the Gro1-4 R gene (R048 and R050 in Figure 5). This confirmed that Gro1-4 was expressed in uninfected roots. Transcripts of members of the Gro1 gene family having a stop codon within the NB domain were also found (gro1-6, gro1-11, and gro1-14). Based on the number of identical RT-PCR products, genes gro1-5 and gro1-6 of the P40 allele had the highest expression level in uninfected roots.

RT-PCR products of the expected size were also generated from stem, leaf, flower, tuber, and stolon cDNA preparations (Figure 6). This showed that members of the Gro1 gene family are expressed in all tested potato tissues.
Discussion

The candidate gene approach for cloning Gro1-4

Positional cloning of a plant gene of unknown sequence requires the construction of a physical map covering the genetic interval between two markers flanking the target gene. This is followed by complementation or mutation analysis of all genes present within the physical map that co-segregate with the targeted gene’s function. This approach is unbiased but usually very laborious and time-consuming, depending on the physical size of the target region, the number of genes to be considered, and the time required for the complementation test. The candidate gene approach has the potential to reduce the number of genes to be considered, but it is biased because of the hypothesis that it makes concerning the identity of the target gene. As the structural similarity was recognized between genes for resistance to different types of pathogens isolated from unrelated plants (Mindrinos et al., 1994), a number of R genes have been cloned by a combination of both approaches, among others the first R genes of potato (Ballvora et al., 2002; Bendahmane et al., 1999; Van der Vossen et al., 2000). In those cases, the sequence analysis of the available physical map revealed candidate genes with structural similarity to other plant R genes, which were then selected and tested for complementation. As the complementation test for resistance to nematodes by stable transformation requires at least 6 months in potato, to limit the number of genes to be tested, a candidate gene approach for cloning the Gro1 gene was adopted, which did not include prior construction of a physical map. The RGL marker Sg22 co-localized with Gro1 based on a high-resolution genetic map (Ballvora et al., 1995; Leister et al., 1996). We hypothesized that the marker Sg22 was, in fact, derived from a gene identical or highly homologous to the nematode R gene. The marker Sg22 identified a complex family of highly homologous candidate genes, which clustered, except for gene gro1-16, at the Gro1 locus. With the exception of gene gro1-9, all candidate genes were subsequently isolated from lambda and BAC genomic libraries. Analysis of inheritance and linkage mapping reduced the number of candidates to five. Sequence analysis of 13 family members revealed putative pseudogenes and further reduced the number of candidates to three genes. The complementation test using these three genes under the control of their endogenous promoter identified the gene Gro1-4, which was able to confer resistance to G. rostochiensis pathotype Ro1 to the susceptible cv. Désirée.

When testing for overlapping ends the BACs having candidate genes derived from the resistant allele of genotype P40, BACs having genes 3 or 8 did overlap, whereas BACs having genes 5, 2, and 11, or 4 and 14 did not overlap with each other or with BACs having gene 6 (unpublished results). Based on BAC insertion sizes between 70 and 100 kbp, and between one and three genes of the Gro1 family in each BAC (Table 1), we estimate that the eight genes are spread over at least 400 kbp. This indicates a genome organization at the Gro1 locus reminiscent of the Mi locus for resistance to the root knot nematode M. incognita on tomato chromosome 6, with six to eight homologous genes present within 650 kbp (Milligan et al., 1998). This is different from the Hero locus for resistance to potato cyst nematodes on tomato chromosome 4, where 14 homologous genes clustered within 118 kbp (Ernst et al., 2002).

Among 1100 plants tested for recombination events in the Gro1 region (Ballvora et al., 1995), only plant R458 was recombinant within the Gro1 gene cluster. Starting with the BACs having candidate genes, the construction of partial BAC contigs did not result in the genetic delimitation of the physical map of the Gro1 region by placement of crossing-over events flanking Gro1 (unpublished data). The physical size of the locus was therefore much larger than previously estimated (Ballvora et al., 1995). The candidate gene approach, although risky, proved to be a useful and effective short cut for cloning Gro1-4.

Structure and function of the Gro1 gene family

The functional Gro1-4 R gene and the other fully sequenced gro1 genes are all members of the TIR/NB/LRR class of plant genes for pathogen resistance. Gro1-4 is the first plant gene for nematode resistance of this type and the first potato gene for resistance to G. rostochiensis. The tomato genes Mi-1.2 for resistance to M. incognita (Milligan et al., 1998) and Hero for resistance to G. rostochiensis (Ernst et al., 2002), and the potato gene Gpa2 for resistance to G. pallida (Bendahmane et al., 1999) are all members of the coiled-coil (CC)/NB/LRR class of plant R genes. Gro1-4 and Hero are functionally most closely related, as both genes confer resistance to pathotype Ro1 of G. rostochiensis. The two genes occupy, in fact, different positions in the highly
syntenic potato and tomato genomes ( Tanksley et al., 1992 ) and share very little sequence similarity. This suggests that they interact with different nematode avirulence factors and may also trigger different signaling pathways. However, gro1-16, the only member of the Gro1 family not included in the gene cluster on chromosome VII, maps to a similar position on potato chromosome IV as does Hero on the syntenic tomato chromosome 4. This can be inferred from similar genetic distances (5–7 cm) of gro1-16 and the tomato marker CT229, most tightly linked to Hero (Ernst et al., 2002), from potato anchor marker GP180 that has been mapped relative to CT229 on the tomato RFLP map (Tanksley et al., 1992). The gro1-16 gene has not been characterized further by sequencing. It may be part of a R gene cluster including different types on the short arm of potato/tomato chromosome 4. In potato, major quantitative trait locus ( QTL ) for resistance to Phytophthora infestans (Leondarts-Schippers et al., 1994) and G. palida (Bradshaw et al., 1998) have been mapped to this genomic region.

At the nucleotide level, Gro1-4 was more than 90% identical with two NB-LRR-type R gene homologs of tomato, which map to syntenic positions on tomato chromosome 7 and are tightly linked to the tomato I3 gene for resistance to Fusarium oxysporum (Bournival et al., 1989; Pan et al., 2000). The I1 gene for resistance to a different race of the same fungus also maps to this region (Sarfatti et al., 1991). The potato Gro1 and tomato I3/11 loci may be orthologous, encoding genes that are highly sequence related but confer resistance to different pathogens such as nematodes and fungi.

The deduced Gro1-4 resistance protein differs from the gro1-2 and gro1-5 susceptibility proteins by 16 non-conservative amino acid changes. One is located between the TIR and NB domains, six are in the LRR domain, and nine in exon IV with unknown functional significance (Figure 4c). One or more of these 16 amino acids may be responsible for the functional difference between Gro1-4 versus gro1-2 and gro1-5. Alternatively, sequence polymorphisms in the putative signal peptide region and further upstream in the promoter region may alter transcription, translation, and/or transport of gro1 polypeptides, rendering them non-effective. One such candidate for functionally relevant polymorphisms is a microsatellite sequence of variable length that is located within 300 bp upstream from the second translation start codon of gro1 genes.

Preliminary data indicate that Gro1-4, unlike Hero (Ernst et al., 2002), does not confer resistance to all pathotypes of G. rostochiensis. In contrast, P40, the source of the Gro1-4 gene, carries broad-spectrum resistance to G. rostochiensis (Barone et al., 1990). The possibility exists that other members of the Gro1 gene family are also functional nematode R genes and confer resistance to pathotypes other than Ro1. The broad-spectrum resistance phenotype of P40 could then result from the concerted action of several family members. This is subject to further studies.

Expression of the Gro1 gene family

Despite the fact that Gro1-4 contains a large retroelement in intron I, Gro1-4 is expressed at low level in uninfected roots of nematode-resistant plants, together with several other members of the gene family, including putative pseudogenes. The expression of genes that contain retroelements has been reported. The gene encoding starch branching enzyme (SBEIIb) of barley contains a retrotransposon-like element in intron II (Sun et al., 1998). The intragenic insertion of a retrotransposon can result in alternative RNA processing, as has been shown for the maize waxy gene (Marillonet and Wessler, 1997). Transcripts smaller than expected for genes Gro1-4, gro1-2, and gro1-5 were detected on a virtual Northern blot using amplified root cDNA and among sequenced RACE products. This indicates occurrence of truncated or alternatively spliced transcripts of members of the Gro1 gene family. Alternative splicing has also been observed for the N gene conferring resistance to tobacco mosaic virus (Whitham et al., 1994).

Members of the Gro1 gene family were expressed in all potato tissues tested. The distribution of gene-specific transcripts was not analyzed in tissues other than roots. Biological functions other than nematode resistance may therefore be encoded by the Gro1 gene family.

Experimental procedures

Plant material

P40, P18, R458, P41, and P6/210 are diploid potato genotypes. P40 (H80.696/4) is an interspecific hybrid between S. tuberosum and S. spagazzini. P40 is heterozygous for the Gro1 R gene and resistent to all five known pathotypes (Ro1–Ro5) of G. rostochiensis (Barone et al., 1990). P18 (H82.337/49) is the susceptible parent of the cross P18 × P40 used to map the Gro1 resistance locus (Ballvora et al., 1995; Barone et al., 1990). R458 is one of 121 F1 hybrids of the cross P18 × P40 that were selected from 1100 gametes of P40 for being recombinant between two markers flanking the Gro1 locus (Ballvora et al., 1995). R458 is heterozygous resistant to G. rostochiensis pathotype Ro1 (Gro1gro1). P41 (H79.1506/1) and P40 are the parents of line P6/210 used to construct the lambda and BAC genomic libraries. P6/210 was selected for carrying both the Gro1 gene for nematode resistance and the R1 gene for late blight resistance in the heterozygous state (Ballvora et al., 2002). The tetraploid cv. Désirée, which is susceptible to all pathotypes of G. rostochiensis was used for complementation assays. Three accesses of the wild tuber-bearing species S. spagazzini are represented by plants spagazzini (spg)12, spg14, and spg15 and were kindly provided by J. R. Hoekstra (Bundesforschungsanstalt für Landwirtschaft (FAL) at Braunschweig, Germany). Resistance of the spg plants to G. rostochiensis has not been tested.
**Genomic libraries and library screening**

A potato BAC library constructed as described by Meksem et al. (2000) from high-molecular weight DNA of line P6/210 was kindly supplied by LION Biosciences AG (Heidelberg, Germany). The library consisted of c. 100,000 clones with an average insert size of 70-kbp potato genomic DNA, cloned into the HindIII site of the pCDL04541 vector (Jones et al., 1992). The library covers the diploid potato genome approximately seven times. The BAC library was screened by hybridization of high-density filters to the 32P-labeled probe St332 (GenBank Accession no. U60080; Leister et al., 1996), as described by Baulorva et al. (2002). A potato lambda library was constructed from genomic DNA of line P6/210 that was partially restricted with MboI and size-fractionated by sucrose density gradient ultracentrifugation using standard procedures (Sambrook et al., 1989). The 15–20-kbp DNA size fraction was ligated into the BamHI pre-digested lambda DASH II vector, packaged using Gigapack II XL packaging extracts, and amplified in Escherichia coli strain XL1-Blue MRA (P2) following the supplier’s instructions (Stratagene, La Jolla, CA, USA). Plaque lifts and filter hybridization to the St332 probe were performed using standard procedures (Sambrook et al., 1989).

**DNA isolation**

Lambda plate lysate stocks and large-scale bacteriophage preparations were generated using standard protocols (Sambrook et al., 1989). Lambda DNA was prepared using Qiagen Lambda Maxi Kit (Qiagen, Hilden, Germany). BAC DNA was isolated as described by Baulorva et al. (2002) by using the Qiafilter Plasmid Purification Kit (Qiagen). Potato genomic DNA was extracted and purified as described by Gebhardt et al. (1989) and Oberhagemann et al. (1999).

**Southern gel blot analysis**

TaqI enzyme restriction digest, electrophoresis on denaturing polyacrylamide gels (4% w/v), electroblotting onto a Biodyne B nylon membrane (Pall, East Hills, NY, USA), and hybridization to the 32P-labeled St332 probe were carried out as described by Gebhardt et al. (1989). Four to five micrograms of potato genomic DNA and 10 ng of BAC or lambda DNA were used per sample. Exposed phosphor imager screens were scanned using a STORM 860 Phosphor Imager (Amersham Pharmacia, Rockville, MD, USA).

**Gene-specific primer markers**

Gene-specific primers were designed, either on the basis of anonymous end sequences from BAC or lambda clone genomic inserts containing the corresponding TaqI fragment (Table 1) or on the basis of sequences specific for the internal St222 homologous gene. Gene-specific sequence variation was identified by sequence alignment and comparison of all members of the St222 gene family located at the Gro1 resistance locus of P40. PCRs were carried out in 25 μl volume on a PTC-225 Tetrad thermocycler (MJ Research, Waltham, MA, USA) using the following conditions: 4 min at 94°C followed by 40 cycles of 1 min at 92°C, 1 min at 58°C and 1 min kbp-1 at 72°C, and finally 10 min at 72°C. PCRs consisted of buffer (Invitrogen, Carlsbad, CA, USA), 10 μM primers A and B (Table 2), 0.5 μl Taq DNA polymerase (Invitrogen, Carlsbad, CA, USA), 1× BSA, 2% DMSO, 0.2 mM dNTPs, 1.4 mM MgCl2 and 50 ng genomic, 5 ng BAC, or 1 ng lambda DNA as template.

**Linkage mapping**

Ninety-two F1 hybrids of the cross P18 × P40 (mapping population F1940, Leister et al., 1996) were scored for presence or absence of polymorphic PCR products. Linkage analysis relative to mapped RFLP markers was performed as described by Gebhardt et al. (1989) using the MAPRF software package (E. Ritter, NEIKER, Vitoria, Spain).

**RACE and RT-PCR**

Total RNA was isolated from different potato tissues using the RNeasy Plant Mini Kit (Qiagen) and the RNase-Free DNase Set (Qiagen) following the manufacturer’s protocols. Tissues with high starch content (tubers and stolons) were treated with Concert Cyttoplasmic RNA Reagent (Invitrogen) after powderization under liquid nitrogen. For the virtual Northern blot, total root RNA was isolated from P6/210 20 days after infection with G. rostochiensis pathotype Ro1. Double-stranded cDNA was synthesized with the SMART library construction kit (Clontech, Palo Alto, CA, USA) according to the supplier’s instructions. Ten micrograms of cDNA were separated on 1% agarose gel in 1× 40 mM Tris-acetate 1 mM EDTA (ethylene-diamin-tetra-acetate) (TAE) buffer, blotted, and probed with the 32P-labeled St332 sequence.

For RT-PCR experiments, double-stranded cDNA was generated from total RNA of different tissues of uninfected P6/210 plants and amplified using the SMART RACE cDNA Amplification Kit (Clontech). Primers for RT-PCR had the following sequences: NBSRACE1, 5'-GGA AGA AGA AGA ATT CAG TGA CCA GAA TAC TTG A-3'; LRNRACE1, 5'-CCT TGT AGG TTT TCC AAA GGT GTA TGA TGC G-3'. RACE was carried out using the Marathon cDNA amplification kit (Clontech). RACE products were generated using the following St332 internal primers: For 3' RACE, we used primers gsp1, 5'-GAC GGA AGT AGT ATG ACA ACC C-3' and gsp2, 5'-TGA ATG GTG TGT YGA CGG AAG TAG AAT CAT C-3'; for 5' RACE, we used primers gsp1, 5'-GTT TGT TAT GAT GAT TCT ACT TCC GTC-3' and gsp2, 5'-CCT GTT TGT CGT AAC GGT CTC TGA CTT CGT G-3'. PCR products were cloned using the pGEM-T vector system (Promega, Madison, WI, USA).

**Generation of genomic subclones**

Five hundred nanograms of DNA from BAC clones BA26N18 and BA60B11 were partially digested with the restriction enzyme Tsp560I. Five hundred nanograms of BAC BA98P9 were completely digested with the restriction enzyme BamHI. DNA fragments were separated on a 1% low-melting-temperature agarose gel (Sea Plaque GTG Agarose, Bioproduits, Rockland, ME, USA) using the CHEF-DR III Puls Field Gel Electrophoresis System (Bio-Rad, Hercules, CA, USA). DNA fragments were recovered using the GE lase system (Epicentre Technologies, Madison, WI, USA) and ligated into the pCDL04541 plant transformation vector (Jones et al., 1992), which had been linearized by EcoR I or BamHI and dephosphorylated with shrimp alkaline phosphatase (Roche, Mannheim, Germany). Constructs were transformed into DH10B cells (Invitrogen). Positive clones were identified by colony hybridization with 32P-labeled St332 sequence using standard protocols (Sambrook et al., 1989).

**Generation of transgenic plants**

Competent LBA4404 Agrobacterium cells were prepared and transformed via electroporation with pCDL04541 constructs as
described by McCormac et al. (1998) using a Gene Pulser (Bio-Rad). Transformation in MS medium (Murashige and Skoog, 1962) of leaves of cv. Désirée by Agrobacterium strains and regeneration of transgenic callus on MS medium supplemented with 50 mg l⁻¹ kanamycin and 250 mg l⁻¹ clofaran were carried out as described by Rocha-Sosa et al. (1989). At least 20 independent transgenic lines for each transgene were regenerated.

Test for resistance to G. rostochiensis

Cysts of G. rostochiensis pathotype Ro1 were kindly provided by Dr H. J. Rumpenhorst (Biologische Bundesanstalt, Münster, Germany) and Dr M. Arndt (Bayerische Landesanstalt für Boden­kultur und Pflanzenbau, Freising, Germany). Three to four shoot cuttings of each transgenic line were subcultured for 3–4 weeks on MS medium (Murashige and Skoog, 1962) until small roots formed. Plantlets were then transferred into pots (diameter 6–8 cm) filled with a mixture of 50% soil (Einheitserde) and 50% sand. Each pot was inoculated with 20 cysts. Plants were grown in the greenhouse under normal daylight conditions or in a phytocabinet (BBC-York, Germany), 16 h at 22 °C in the light and 8 h at 18 °C in the dark. The roots were visually scored after 6–7 weeks for the presence of newly formed cysts. Transgenic lines were scored resistant when the roots of all infected plants showed no or very few cysts (up to five) and susceptible when more than five new cysts were visible on the roots. Untransformed Désirée plants were used as susceptible control and P40 plants as resistant control.

Sequence analysis

DNA sequences were determined by Automatische DNS isolierung und Sequenzierung (ADIS), the Max-Planck-Institut für Züchtungsforschung (MPIZ) DNA core facility, on ABI Prism 377 or 3700 automated sequencers (Applied Biosystems, Foster City, CA, USA) using BigDye-terminator chemistry. Pre-mixed reagents were supplied by Applied Biosystems. Oligonucleotides were purchased from Metabion (Martinsried, Germany). Sequences were analyzed using the GCG-software package for UNIX, version 10 (Genetics Computer Group, WI, USA) and the LASERGENE software version 5 (DNASTAR, WI, USA). Sequence homology searches were performed using European Molecular Biology Laboratory (EMBL), National Centre for Biotechnology Information (NCBI), and DNA Data Bank of Japan (DDBJ) database platforms. The SIGNALP program at http://www.cbs.dtu.dk/services/SignalP and the PROFILSCAN server at http://hits.isb-sib.ch/cgi-bin/PSFScan were used for functional prediction. Putative promoter sequences were analyzed with PLACE SIGNAL SCAN Search (Higo et al., 1999; http://www.dna.affrc.go.jp/htdocs/PLACE/). CLUSTALW at http://www.ebi.ac.uk/clustalw was used for multiple sequence alignment, and TREEVIEW was used for tree drawing (Roderick D.M. Page, download from http://taxonomy.zoology. gla.ac.uk/rod/rod.html).

Acknowledgements

This research was supported by the Max-Planck society, by grant no. G-566-269-12.97 from GIF, the German-Israeli Foundation for Scientific Research and Development, and by Bundes Ministerium für Bildung und Forschung (BMBF) grant no. 0311791 from the German Ministry for Education and Research. The technical assistance of Monique Winkler, Birgit Walkemeier, and Iris Schmitz is gratefully acknowledged.

References


Accessions: AY196151–AY196163.