Identification of Resistance Gene Analogs and Verticillium Wilt Resistance-like Sequences in Mentha longifolia

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ABSTRACT. Resistance gene analog (RGA) sequences were obtained from four Mentha longifolia (L.) Huds. accessions using degenerate polymerase chain reaction (PCR) primers targeting the conserved nucleotide binding site domain found in many plant disease resistance genes. Seven distinct RGA families were identified. All M. longifolia RGAs showed similarity to sequences of the non-toll-interleukin 1 receptor R gene class. In addition, degenerate PCR primers based on the tomato (Solanum lycopersicum L.) verticillium wilt resistance (Ve) genes were used to PCR-amplify a 445-base pair (bp) Ve-like sequence from M. longifolia that had ≈57% predicted amino acid identity with Ve. Mint-specific primers based on the original mint Ve sequence were used to obtain mint-specific Ve sequences from four M. longifolia accessions and from peppermint (Mentha piperita L.) cultivar ‘Black Mitcham’ that had 95% to 100% predicted amino acid identity to the original mint Ve sequence. Inverse PCR was then used to obtain flanking Ve sequence from one M. longifolia accession extending the mint Ve sequence to 1077 bp. This is the first report of RGA sequences in the Lamiaceae and the first report of Ve-like sequences obtained with degenerate PCR primers.

We are using Mentha longifolia (Lamiaceae) as a diploid model species with relevance to the polyploid commercial mints (Mentha L. spp.) and to the study of plant resistance to vascular wilt diseases. Verticillium wilt, incited by the fungus Verticillium dahliae Kleb., is the most damaging disease of mint, verticillium wilt resistance, disease resistance, nucleotide binding site, leucine-rich repeat, receptor kinase proteins are predicted or demonstrated to have roles other than plant defense, the NBS-LRR proteins have been implicated solely in plant disease resistance (Meyers et al., 2005). The Arabidopsis thaliana (L.) Heynh. genome includes 149 NBS-LRR sequences (Meyers et al., 2005).

The NBS-LRR superclass can be divided into two classes based on motifs within the ≈200 amino acid zone upstream of the NBS: the toll-interleukin receptor-like (TIR-NBS-LRR) class and the non-TIR class, which has a coiled-coil domain. The TIR domain has homology to the Drosophila melanogaster Meigen toll receptor and the mammalian interleukin-1 receptor, both of which are involved in immune responses (Jebanathirajah et al., 2002). TIR and non-TIR classes of R genes can be distinguished by amino acids within the NBS. The TIR class has motifs of amino acid sequences LQKKLLSKLL (called RNBS-A-TIR) and FLHIACFF (called RNBS-D-TIR), whereas the non-TIR class has FDLXAWVCVSQXF (called RNBS-A-non-TIR) and CFLYCALFPED (called RNBS-D-non-TIR) (Meyers et al., 1999). In addition, the presence of tryptophan (W) or aspartic acid (D) at the final amino acid position of the Kin-2 motif distinguishes the non-TIR and TIR classes, respectively, with 95% accuracy (Meyers et al., 1999).

The NBS itself, a protein domain represented in both prokaryotes and eukaryotes, contains highly conserved motifs. The most common NBS motif is the “P-loop/kinase 1a” domain involved in binding ATP or GTP (Saraste et al., 1990; Traut, 1994). NBS domains of plant R genes include the P-loop as well as Kin-2, RNBS-A-D, and GLPL motifs (Meyers et al., 1999). R gene analogs (RGAs) are sequences sharing these conserved NBS motifs. Such NBS motifs were first exploited as target sites for degenerate primer polymerase chain reactions (PCRs) for the purpose of isolating families of RGAs by Kanazin et al. (1996), Leister et al. (1996), and Yu et al. (1996). Degenerate primers have since been used extensively to identify RGA sequences and candidate R genes in numerous angiosperms (Brotman et al., 2002; Cordero and

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Skinner, 2002; Irigoyen et al., 2004; Lopez et al., 2003; Maleki et al., 2003; Martinez Zamora et al., 2004; Noir et al., 2001; Shen et al., 1998; Soriano et al., 2004; Yaish et al., 2004), gymnospersms in the Pinus L. genus (Diaz and Ferrer, 2003; Liu and Ekramoddoulah, 2003), and even the bryophyte Physcomitrella patens (Hedw.) B.S.G. (Akita and Valkonen, 2002).

RGAs are one of two categories of mint candidate gene sequences acquired using degenerate primers in the present study, the second being putative homologs of the tomato Ve (verticillium wilt resistance) genes as described subsequently.

Verticillium dahliae infects a large number of important crop plants, including tomato (Solanum lycopersicum L.), potato (Solanum tuberosum L.), pepper (Capsicum L.), cotton (Gossypium L.), strawberry (Fragaria L.), lettuce (Lactuca L.), and melon (Cucumis L.) (Bhat and Sabbarao, 1999) as well as trees and woody and herbaceous ornamental crops (Sinclair et al., 1987; Smith and Neely, 1979). Verticillium wilt results from fungal invasion of the plant vascular system. For some plant hosts, variability in resistance has been identified in germplasm, but monogenic determinants of resistance have not been reported. The single exception is tomato for which single-gene verticillium wilt resistance was identified in classical genetics experiments and was designated Ve1 (Schiable et al., 1951). This resistance has been bred into many modern tomato cultivars and has proven durable. The Ve locus was eventually mapped to tomato linkage group IX (Diwan et al., 1999). Positional cloning then determined that this locus consisted of two functional genes, encoding products with 84% amino acid identity, designated Ve1 and Ve2 (Kawchuk et al., 2001).

The S. lycopersicum verticillium wilt resistance genes Ve1 and Ve2 (to be collectively referred to as Ve) are predicted to encode cell surface receptor-like proteins that lack an NBS but have 38 LRRs, an N-terminal leucine-zipper motif, and endocytosis-like signals (Kawchuk et al., 2001). Transfer of Ve transgenes to susceptible tomato and potato lines has conferred resistance to race 1 of V. dahliae (Kawchuk et al., 2001). Library probes and PCR primers specific to the tomato Ve sequences have been used to identify homologous sequences from other solanaceous species (Chai et al., 2003; Simko et al., 2004). Outside the Solanaceae, only a few sequences with homology to Ve1 and Ve2 have been listed in the National Center for Biotechnology GenBank database, none of which have been shown to play a role in verticillium wilt resistance. However, the cloning of the tomato Ves has opened up the opportunity to use their sequences and those of possible homologs in other species as a basis for design of degenerate primers aimed at isolating Ve homologs in mint.

Here we report the use of degenerate primer PCR for identification of two types of disease resistance-related sequences in M. longifolia: 1) RGAs, which may represent a variety of disease specificities; and 2) sequences homologous to the known verticillium wilt resistance (Ve) genes of tomato.

Materials and Methods

Plant and fungal material. Mentha longifolia accessions were obtained as stolons or rooted cuttings from the USDA-NCGR, Corvallis, OR. Plants were maintained in a greenhouse at the University of New Hampshire, Durham, in 22-cm-diameter pots and propagated vegetatively. Accessions CMEN17.001 (PI 557755), CMEN585.001 (PI 557767), CMEN501.001 (PI 212314), and CMEN81.001 (PI 557759) were previously classified as verticillium wilt-resistant, whereas accessions CMEN584.001 (PI 557769) and CMEN516.001 (PI 557760) were classified as wilt-susceptible (Vining et al., 2005). For convenience, the “.001” suffix common to all of these CMEN accession numbers is dropped throughout the subsequent text. Peppermint cultivar ‘Black Mitcham’, classified as verticillium wilt-susceptible (Douhan and Johnson, 2001), was also used. The accessions used in this study are diploid (2n = 2x = 24) with the exceptions of CMEN501 (tetraploid: 2n = 4x = 48), CMEN81 (unknown ploidy), and ‘Black Mitcham’ (hexaploid: 2n = 6x = 72) (Chambers and Hummer, 1994). The V. dahliae culture used to generate inoculum was provided by D. Johnson (Washington State University, Pullman, WA).

Molecular techniques. Genomic DNA was extracted from fresh, unexpanded leaf tissue using a modified CTAB miniprep method as described in Vining et al. (2005). For reverse transcriptase (RT)–PCR, template RNA was obtained from roots of plants that had been incubated in either water or an aqueous V. dahliae spor suspension (≈10⁶ spores/mL) for 16 to 24 h. Total RNA was isolated from 100 to 200 mg fresh or frozen roots using Trizol reagent according to the manufacturer’s instructions (GIBCO, Carlsbad, CA). Messenger RNA (mRNA) was separated from total RNA using oligo d(t) cellulose columns (GIBCO).

All PCR conditions are listed in Table 1. Reaction volumes of 25 μL contained 100 ng template DNA, 0.1 U Taq DNA polymerase (Sigma, St. Louis), 2.5 mM each dNTP (Promega Corp., Madison, WI), and either 4.8 μmol of each degenerate primer or 0.8 μmol of each specific primer. RT-PCR was performed with the ProSTAR HF Single-Tube RT-PCR System (Stratagene, La Jolla, CA). Inverse PCR (IPCR) was used to obtain sequences flanking the mint Ve segment initially identified by degenerate primer PCR. One microgram of total genomic DNA from CMEN585 was digested overnight at 37 °C in a 20-μL reaction with 10 U of the restriction enzyme MseI (New England Biolabs, Ipswich, MA). Two microliters of the

| PCR profile      | PCR primer set | Denaturation step | Annealing step | Extension step | Cycles (no.) |
|------------------|----------------|-------------------|----------------|---------------|--------------|
| Degenerate       | 1 + 2, 1 + 3, 4 + 5 | 94 °C 50 s         | 56 °C 1 min 20 s, 45 °C 30 s | 72 °C 1 min    | 34           |
| Reverse transcription | 1 + 2       | 95 °C 1 min       | 56 °C 1 min 20 s, 45 °C 30 s | 68 °C 2 min    | 39           |
| Specific         | 6 + 7, 8 + 9, 10 + 11 | 94 °C 1 min       | 55 °C 1 min 20 s, 45 °C 30 s | 72 °C 1 min    | 30           |
| Inverse          | 12 + 13      | 94 °C 1 min       | 58 °C 1 min 20 s, 45 °C 30 s | 72 °C 2 min 30 s | 30           |

*Denegenerate, specific, and inverse polymerase chain reaction (PCR) profiles had an initial 2 min, 94 °C denaturation step and a final 7 min, 72 °C extension step. The reverse-transcription PCR (RT-PCR) profile had an initial, first-cDNA-stand synthesis step at 37 °C for 45 min and a final extension at 68 °C for 10 min.

Table 1. Polymerase chain reaction conditions used for amplification of resistance gene analog (RGA) and verticillium wilt-resistance-like (Ve) sequences from Mentha longifolia (L.) Huds.*

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digested were used directly in a ligation reaction with 400 U Taq DNA ligase (New England Biolabs) to circularize the genomic restriction fragments. The ligation reaction was incubated for 2.5 h at room temperature and then stored at 4 °C overnight. PCR was performed in 25-μL reactions using 5 μL of the ligation products as template, 0.1 U Taq polymerase (Sigma), 2.5 mM of each dNTP (Promega), and 0.8 umol of each inverse primer.

Widely used degenerate PCR primers targeted to the P-loop motif (forward primer s2) and hydrophobic domain (reverse primers as2 or as3) (Leister et al., 1996) were used for initial amplification of RGAs from genomic DNA (gDNA) and from cDNA produced by RT-PCR (Table 2). Degenerate primers intended to amplify Ve homologs in mint were designed manually on the basis of tomato Ve1 amino acid and nucleotide sequences aligned with the most similar respective sequences from A. thaliana and Oryza sativa L. obtained by searching GenBank’s nonredundant (nr) database using the blastx algorithm (Altschul et al., 1990) submitting the tomato Ve1 amino acid sequence as a search query. Subsequently, specific (nondegenerate) primers intended to amplify segments of putative mint Ve homologs or a 425-base pair (bp) segment of the tomato Ve1 gene itself (as a positive control) were designed using PrimerSelect software (version 5.53; DNAStar, Madison, WI). The tomato-specific forward and reverse primers, Ve5F19 and Ve5R23, bracketed Ve1 nucleotides 1776 to 1794 and 2200 to 2178, respectively (Table 2).

PCR, RT-PCR, and IPCR products were electrophoretically separated on 2% agarose gels in 1 X TBE, pH 8.0 at 4.5 V/cm at room temperature. Gels were stained with ethidium bromide and photographed over ultraviolet light. Amplification products were recovered from gel slices using the Geneclean Spin kit (QBiogene, Irvine, CA) and cloned with the TOPO TA Cloning kit (Invitrogen, Carlsbad, CA). Recombinant plasmids were isolated using the Wizard SV Plasmid Purification kit (Promega), and photographed over ultraviolet light. Amplification products were electrophoretically separated on 1% agarose gels in 1 X TBE, pH 8.0 at 4.5 V/cm at room temperature. Gels were stained with ethidium bromide and photographed over ultraviolet light. Amplification products were recovered from gel slices using the Geneclean Spin kit (QBiogene, Irvine, CA) and cloned with the TOPO TA Cloning kit (Invitrogen, Carlsbad, CA). Recombinant plasmids were isolated using the Wizard SV Plasmid Purification kit (Promega).

Cycle sequencing reactions were performed following the University of New Hampshire’s Hubbard Center for Genome Studies protocol (Hubbard Center for Genome Studies, University of New Hampshire, 2002) using DYEnamic Terminator ET cycle sequencing premix (Amersham Biosciences, Piscataway, NJ) and standard plasmid vector primers M13F, M13R, T3, and T7. Reaction products were analyzed using an ABI PRISM 377 DNA sequencer (Applied Biosystems, Foster City, CA).

**Sequence analysis.** DNA sequence chromatograms were viewed and assessed for base-calling accuracy using SeqEd version 1.0.3 (DNASTAR). Similarity searches of the GenBank nr database were conducted using blastx and tblastx algorithms (Altschul et al., 1990). Clustal X (Thompson et al., 1997) was used to perform sequence alignments and to generate an amino acid sequence identity matrix of the RGAs, including top non-mint RGA matches from the GenBank nr database (RGA blast hits). A Clustal X alignment of translated mint RGAs and top blast hits was used to construct a neighbor-joining tree with 1000 bootstrap replicates.

**Results**

**Resistance gene analogs.** The insert sizes of 52 PCR product clones obtained from genomic DNA of four verticillium wilt-resistant M. longifolia accessions using degenerate RGA primers (s2 + as2 or s2 + as3) ranged from 289 to 680 bp. When these sequences were subjected to blastx and tblastx searches of the GenBank nr database, 20 of them had best matches (i.e., blast hits) that were R genes or RGAs. These M. longifolia genomic RGA sequences are referred to as gRGAs (Table 3). Of the 20 gRGA sequences acquired, seven from CMEN585, six from CMEN17, three from CMEN81, and one from CMEN501, 17 were found to be unique after discounting sequence redundancy. One gRGA sequence was represented by three identical clones: two from CMEN585 (clones 5856 and 58531) and one from CMEN17 (clone 1744).

| Primer name | Target sequence | Primer sequence (5‘ to 3’)
|-------------|-----------------|-----------------------------|
| 1. s2 (forward) | P-loop (GGVGKTT) | GGNGNGTNGGNAANACNAC |
| 2. as2 (reverse) | Hydrophobic domain (GLPLAL) | NAAAGCNAGNNGNAANCC |
| 3. as3 (reverse) | Hydrophobic domain (GLPLAL) | NAGNCGNAGNNGNAGNCC |
| 4. Ve5F19 (forward) | Tomato Ve1-specific | GAGATACGTTGGAACAGCCT |
| 5. Ve5R23 (reverse) | Tomato Ve1-specific | AGTTATTCGCACGTAGGTTCAAT |
| 6. Ve2398–2416F Actual sequence at primer site in CMEN585 | Ve LRR | ATYNNTGATATAGCYYCTCAA (ATCATCGATATAGCYYCTCAA) |
| 7. Ve2791–2810R Actual sequence at primer site in CMEN585 | Ve LRR | CTGTGGRATYTCYCC (ATTCGGGATCTCTCC) |
| 8. Ve4–24F Actual sequence at primer site in CMEN585 | Mint Ve | GTCGATAGCTTCCCAAAAT (ATCGATATATCTTCCCAAAAT) |
| 9. Ve403–386R | Mint Ve | TCAGGTACATGCCCCTGACA |
| 10. Ve111–133F | Mint Ve | TACGCGCGCTTGTCCGCTTCTT |
| 11. Ve974–951R | Mint Ve | TCAGCGCGCGCTTGTCCGCTTCTT |
| 12. IPCR138–153F | Mint Ve | GAAAGATCCCGAAGT |
| 13. IPCR325–304R | Mint Ve | CAGCTAGAGAAGTTTATTGATT |

Table 2. Oligonucleotide primers used to amplify resistance gene analog (RGA) and verticillium wilt resistance-like (Ve-like) sequences from Mentha longifolia. * Primers s2, as2 and as3 are from Leister et al. (1996). All other primers were designed during the present study for amplification of M. longifolia Ve-like sequences.
Another gRGA was represented by two identical clones, 1712 and 1725, both from CMEN17.

All but three of the 17 unique gRGA sequences consisted of uninterrupted open reading frames (ORFs). Clone 5857 had an RGA reading frame that was interrupted by one stop codon [position 271–273 (TAG)], and clone 171 had an RGA reading frame interrupted by two stop codons [positions 229–231 (TAA) and 358–360 (TAA)]; otherwise, both of these interrupted RGA sequences were continuous ORFs. gRGA clone 173 had an RGA sequence that shifted the reading frame forward one nucleotide at a point 245 bases into the sequence dividing two nearly continuous ORFs that were each interrupted by a single stop codon [positions 79–81 (TGA), 386–388 (TGA)].

The s2 + as2 degenerate primer combination was also used in RT-PCR with mRNA from both inoculated and noninoculated roots. After RT-PCR, the most densely staining area of the gel lane (in the 250- to 500-bp range), or a discrete gel band, if present, was excised for cDNA recovery. Among 23 sequenced cDNA clones, seven RGAs were identified (Table 3). These cDNA-derived RGAs are referred to as cRGAs. All seven cRGAs were comprised of uninterrupted ORFs. Of the cRGAs from noninoculated root tissue, one, two, and three cRGAs, respectively, were from CMEN17, CMEN585, and CMEN81. Clone 17B9 from CMEN17 was the only cRGA recovered from inoculated roots. The sequence of cRGA clone 1719 was identical to that of gRGA clone 58513 from CMEN585.

Of 31 genomic and 16 cDNA non-RGA sequences obtained using the degenerate RGA primer pairs, one cDNA and four genomic sequences had ABC transporter sequences as best blast hits, and three cDNA and two genomic sequences’ best blast hits were gag-pol or Ty3/gypsy retroelements. Among the other cDNA sequences that had uninterrupted ORFs, blast matches included one chlorophyll a/b binding protein, one L29 ribosomal protein, and two different “senescence-associated proteins.” The rest of the non-RGA genomic and cDNA clones lacked uninterrupted ORFs and had no informative blast hits.

Among a total of 27 gRGA and cRGA sequences acquired using the degenerate RGA primer pairs, there were 23 unique RGA sequences, all of which have been submitted to GenBank (under accession numbers shown in Table 3). All 23 unique mint RGAs contained the RNBS-A non-TIR motif, and 20 of them had tryptophan in the terminal position of the Kin-2 motif, which is characteristic of the non-TIR RGAs. When each of the 23 unique mint RGAs was used in blastx and tblastx searches of the GenBank non redundant (nr) database, the best blast hits were always mint RGAs from the present data set; but many very good nonmint blast hits were also obtained. In many instances, the same best non-mint blast hit was obtained for two or more mint RGAs.

The 23 unique mint RGAs and a selection of 14 GenBank sequences chosen to represent the diversity of top nonmint blast hits were subjected to a Clustal X analysis that generated an amino acid sequence alignment (Fig. 1) and identity matrix
Percent amino acid sequence identities of pairs of unique mint RGAs ranged from 18% to 96%. From the Clustal X alignment, a neighbor-joining tree was generated, which organized the 23 mint RGA sequences and best nonmint blast hits into three major groups (I, II, III) that were further subdivided into seven distinct families (A through G) (Fig. 2). Percent amino acid identities of mint sequences within families ranged from 48% to 96%. The two most similar pairs of sequences were in family E, in which gRGA sequences 501AS38A and cRGA 81CRGC4 were 96% identical, and in family B, in which gRGA sequences 5856 and 178 were 89% identical. Mint RGAs in family G were most similar to Phytophthora infestans (Mont.) de Bary R genes from Solanum L. species, whereas mint RGAs in families C and D were most similar to potato and pepper R genes with known pathogen specificities (Table 4). For mint RGAs in families B, E, and F, representatives from several angiosperm species were included among the top nonmint blast hits (Table 4). Top non-mint blast hits for mint family A were RGAs from Coffea L. species.

When a blastx search of the GenBank nonredundant database was performed using the tomato Ve1 amino acid sequence as a search query, the most similar sequences (30% to 96%) were from Phytophthora infestans, Solanum L. species, and other plant species (Table 4).
40% amino acid identities) were those from A. thaliana (NP_187712, NP_198058, NP_180861, NP_187217) and O. sativa (BAA96753, BAB08209). Using both amino acid and nucleotide alignments of the tomato Ve1 and Ve2 sequence with the aforementioned A. thaliana and O. sativa sequences as a basis for identifying conserved regions, degenerate primers Ve2398–2416F and Ve2791–2810R (Table 2) were designed manually. These primers, which targeted sites corresponding to codons 800–805 and 942–948 of tomato Ve1, amplified a 445-bp product from CMEN585, the sequence of which we designate the “original mint Ve.” Excluding from consideration the amino acids coinciding with the primer sites, the original mint Ve predicted amino acid sequence (mint orig) shared 57.1% and 56.5% amino acid identity with the corresponding regions of the tomato Ve1a and Ve2 sequences, respectively (Fig. 3).

On the basis of the original mint Ve sequence, mint-specific primers (Ve4–24F and Ve403–386R; Table 2) internal to the for design of specific PCR primers (Ve111–133F and Ve974–951R; Table 2), which bracketed 865 nucleotides of the extended mint Ve sequence. The predicted amino acid sequence of the extended mint Ve aligned with the C terminus of the tomato sequences, spanning amino acids 730 to 1053 and 728 to 1079 of Ve1 and Ve2, respectively (Fig. 3). This section of the tomato sequences included the following regions defined by Kawchuk et al. (2001): LRRs 28 to 38 (end of region B); negatively charged extracytoplasmic (C); membrane-spanning (D); and positively charged cytoplasmic (E). Although the extended mint Ve sequence shared ≈57% amino acid identity over the corresponding B region of the tomato Ve sequences, the mint sequence shared only seven of 21 and eight of 21 amino acid identities with the D regions of Ve1 and Ve2, respectively. The C and E regions of the mint and tomato Ve sequences could not be meaningfully aligned.
Table 4. Closest matches (blast hits) for mint resistance gene analog (RGA) sequences obtained by blastx searches of the GenBank nonredundant database.

| Mint RGA family | Top blast hits (GenBank accession no.) | Plant species                          | Confers resistance to               | Amino acid identity (%) |
|-----------------|----------------------------------------|----------------------------------------|------------------------------------|------------------------|
| A               | Disease resistance-like protein (CAC82603) | Coffea arabica L.                       |                                    | 48                     |
| B               | NBS-LRR-like (AA089149)                 | Gossypium barbadense L.                 |                                    | 41                     |
|                 | RGA Pt19 (AAN08179)                     | Citrus grandis Osbeck × Poncirus trifoliat L. (L.) Raf. |                                    | 43                     |
| C,D             | Putative RGA (AAM77267)                 | Capsicum chacoense Steud.               |                                    | 44                     |
|                 | Bs2 (AAF09256)                          |                                        |                                    |                        |
|                 | Rx (CAB50786)                           | Solanum tuberosum L.                   | Potato virus X                     | 44                     |
|                 | Gpa2 (AAF04603)                         |                                        | Globodera Padilla (Stone) Behrens  | 52                     |
| E               | Disease resistance-like protein (CAC82598) | C. arabica                             |                                    | 40                     |
| F               | NBS-LRR protein (AAZ07904)              | Ipomoca batatas (L.) Lam.              |                                    | 37                     |
|                 | NBS-LRR protein (AAZ07913)              | L. batatas                             |                                    | 53                     |
|                 | RGA (AAL30111)                          | Solanum phureja Juz & Bukasov × Solanum stenotomum Juz & Bukasov | 49 |
|                 | Kinase/encodes NBS (CAC79996)           | Solanum pinnatisectum Dunal            |                                    | 51                     |
| G               | RPI (AAR29069)                          | Solanum bulbocastanum Dunal            | Phytophthora infestans (Mont.) de Bary | 52          |
|                 | RGA1 (AAR29070)                         |                                        |                                    | 53                     |

*Each listed sequence is represented on the neighbor-joining tree (Fig. 2). RGA family designations refer to those in Figure 2.*

**Discussion**

**Resistance gene analogs.** We used degenerate primers targeting the NBS motif in genomic PCR and RT-PCR to identify a set of 23 unique RGA sequences from *M. longifolia*, of which 20 had uninterrupted ORFs. Best blast hits for these RGAs included angiosperm *R* genes with a variety of known disease specificities. The 40% to 50% range of amino acid identities to known *R* genes compares with that seen for RGAs identified using the same degenerate primers in other plant taxa such as strawberry (Martinez Zamora et al., 2004) and coffee (Coffea arabica L.) (Noir et al., 2001). The presence of uninterrupted ORFs in 20 of the 23 unique RGAs is suggestive that at least some of these mint RGAs are derived from functional genes. One gRGA, clone 1719 from CMEN17, was identical to a cRGA (clone 58513) from CMEN585, indicating that this particular RGA is both highly conserved and is expressed, strongly suggesting that it may encode a functional product involved in disease resistance.

Two of the 23 *M. longifolia* gRGA sequences had stop codons in otherwise uninterrupted ORFs, and one gRGA contained a frameshift that separated two nearly continuous ORFs. Similarly, Martinez Zamora et al. (2004) identified 51 RGAs in strawberry, of which 28 had uninterrupted ORFs and 23 had stop codons or frameshifts. In pine (*Pinus monticola* Dougl. ex. D. Don), Liu and Ekramoddoullah (2003) found that 18 of 46 genomic RGA clones and 14 of 21 cDNA RGA clones had at least one stop codon or frameshift. RGAs with interrupted ORFs may be parts of pseudogenes. Plant *R* genes have been shown to occur in complex clusters of related functional genes, pseudogenes, gene fragments, and transposable elements (Lescot et al., 2004; Wei et al., 2002). Whether pseudogenes are evolutionary remnants—once-active genes rendered inactive by accumulation of mutations—or whether they have some function, regulatory or otherwise, remains to be determined on a case-by-case basis.

In two instances, identical RGA sequences were amplified from different accessions. The gRGA clone 1744 from CMEN17 was identical to gRGA clones 5856 and 58513 from CMEN585, whereas gRGA clone 1719 from CMEN17 was identical to cRGA clone 58513 from CMEN585. The amplification of identical RGAs from different accessions suggests that the respective pairs of sequences come from orthologous genes. Four of the mint gRGAs obtained using degenerate primers were represented by multiple clones from the same accession. One pair of identical clones (1725, 1712) was from CMEN17, and another (5856, 58531) was from CMEN585. These identities are probably the consequence of redundant sampling of the same gene. The recoveries of nonidentical RGA sequences from a single accession may result from sampling of different genes or different alleles of the same gene. The closest nucleotide sequence match between nonidentical gRGAs within an accession was 79.8% (clones 8120 and 8116), which is low enough to suggest that these are different genes.

NBS-LRR genes comprise significant proportions of plant genomes. Whole genome sequences are available for: *A. thaliana* ecotype Col-0 and *O. sativa* ssp. *japonica* L. cv. Nipponbare. The *A. thaliana* genome of ≈157 Mb (Bennett and Leitch, 2005) contains 149 NBS-LRR genes (55 non-TIR, 94 TIR), representing ≈1% of the genome (Meyers et al., 2005). The *O. sativa* haploid genome size is estimated to be 389 Mb (International Rice Genome Sequencing Project, 2005). *O. sativa* has ≈500 NBS-containing RGA sequences, all of which are of the non-TIR type, and which, like *A. thaliana*, represent ≈1% of the genome (Monosi et al., 2004). The *M. longifolia* genome size of ≈400 Mb is comparable to that of rice (*O. sativa*) (Bennett and Leitch, 2005). If the total
number of *M. longifolia* RGAs parallels the ≈500 of rice, then the 23 RGAs reported here represent less than 5% of the potential *M. longifolia* RGAs. The low level of redundancy encountered among the *M. longifolia* RGAs is also indicative that these sequences represent a small subset of the RGAs in the mint genome.

Studies of resistance gene clusters have revealed evidence of duplication, recombination, and rearrangement events leading to the expansion of clusters, growth of multigene families, and the potential for evolution of new resistance specificities (Chin et al., 2001; Graham et al., 2002; Kruijt et al., 2004). The 23 *M. longifolia* RGAs grouped into seven distinct families, each of which had identities with different RGAs and *R* genes from other plant species. Particular amino acid motifs conserved within mint RGA families may indicate some functional specificity. However, closely related *R* genes do not necessarily confer resistance to the same pathogen. The potato *Gpa2* and *Rx1* genes are in the same cluster on potato chromosome 12 and are considered to be highly homologous but have very different specificities; *Gpa2* is a nematode (*Caenorhabditis elegans* Miers) resistance gene, whereas *Rx1* confers resistance to a virus (van der Vossen et al., 2000). The *M. longifolia* RGA families C and D each have significant similarity to *Gpa2* and *Rx1*. It would be interesting to determine whether family and *D* mint RGAs map to a common genomic region or cluster like their apparent counterparts do in potato.

All the *M. longifolia* RGAs amplified with degenerate primers based on the P-loop and GLPL domains, or amplified with mint RGA-specific primers, had motifs consistent with the non-TIR class. This result does not lead to a conclusion that TIR-class RGAs are absent from the *M. longifolia* genome. In general, dicot species have been found to contain varying ratios of TIR and non-TIR classes of RGAs, whereas in monocot species, only non-TIR RGA sequences have been observed (Cannon et al., 2002). However, dicot RGA sequences obtained from degenerate primer PCR have not given any indication that non-TIR and TIR classes are amplified in proportions that reflect their presence in plant genomes. The same P-loop and GLPL degenerate primers used in the present study, or very similar primers, amplified both non-TIR and TIR classes of RGAs in alfalfa (*Medicago L.*) (Cordero and Skinner, 2002), but only non-TIR sequences from coffee (Noir et al., 2001). In soybean [*Glycine max* (L.) Merr.], in which Kanazin et al. (1996) found only TIR-class RGAs using P-loop and GLPL-based primers, Peñuela et al. (2002) amplified non-TIR RGAs with a set of degenerate primers pairing a P-loop forward primer with a reverse primer based on the non-TIR RNBS-D motif (CFLYCALFPED). It is possible that the P-loop/GLPL degenerate primer set is biased toward TIR-class or non-TIR-class sequences in a species-dependent manner. The fact that only one RGA was obtained using the as3 reverse primer may also reflect primer bias.

Although the isolation of non-RGA sequences among the PCR products generated by degenerate RGA primers seems inevitable, many RGA reports do not mention whether any non-RGA sequences were obtained with degenerate RGA primers. In the present study, two types of non-RGA sequences were common. Use of degenerate RGA primers generated seven PCR products that had high identity to transposable element-like sequences. Sequences with identity to transposable elements have been mentioned in a few RGA reports (Noir et al., 2001; Timmerman-Vaughan et al., 2000). Five of our mint degenerate primer PCR products had high identity to ABC transporter sequences. Joyeux et al. (1999) amplified an ABC transporter-like sequence from *Brassica napus* L. using degenerate primers nearly identical to s2 and as2. Amplification of ABC transporter sequences with NBS degenerate primers is not surprising, because ABC transporters have an NBS motif similar to that of NBS-LRR disease resistance genes (Theodoulou, 2000). If the isolation of non-RGA sequences was detailed in more
reports of degenerate RGA primer studies, potentially useful insight could be gained into commonalities and patterns of occurrence of such sequences in different species.

Ve. Because Ve is not an R gene of the NBS-LRR class, it would not be expected to be amplified by NBS-targeted degenerate primers such as those used to amplify RGAs. We used degenerate primers targeting part of the tomato Ve LRR region to isolate an original Ve-like M. longifolia sequence of 445 bp from the verticillium wilt-resistant accession CMENS85. The ≈57% amino acid identity of this sequence to the corresponding regions of the tomato Ve1 and Ve2 sequences is higher than that for any other Ve-like sequence found in GenBank. Design of mint-specific IPCR primers enabled us to extend the acquisition of mint Ve sequence to a 1077-bp segment that corresponds to the distal B region and the C, D, and E regions of the tomato Ve genes as described by Kawchuk et al. (2001). To date, this is the first reported use of degenerate PCR primers to isolate a Ve-like sequence and the first targeted isolation of such a sequence outside of the Solanaceae.

Specific primers based on the original mint Ve sequence detected Ve homologs in several wilt-resistant and wilt-susceptible M. longifolia accessions, and from wilt-susceptible cultivar ‘Black Mitcham’, indicating that this primer pair and others based on the extended Ve sequence will be a valuable resource for use in a broader assessment of the diversity of Ve sequences among Mentha accessions and species.

Thus far, no verticillium wilt-related phenotypes have been associated with any of the Ve-like sequences reported in O. sativa or A. thaliana. The potential rice Ve homologs have been listed as such in GenBank, based on inference, as a consequence of the annotation of the O. sativa genome, but monocot species are not known to be hosts for Verticillium Nees fungi. To date, no expression or other studies have been performed to elucidate any connection between rice Ve homologs and nonhost resistance to verticillium wilt. For A. thaliana, differences in symptom development such as stunting and early flowering have been observed, but no verticillium wilt-resistant ecotypes have been identified (Steventon et al., 2001; Veronese et al., 2003). The latter researchers identified a locus (but not a gene) that was correlated with less severe symptom development in ecotype C-24, but they noted that disease development was difficult to parse from normal plant developmental and senescence features. Distinctly verticillium wilt-resistant and -susceptible M. longifolia accessions have been described (Vining et al., 2005), making this a much more appropriate species than either O. sativa or A. thaliana for identification and characterization of Ve homologs and assessment of their possible roles in conferring verticillium wilt resistance. As yet, the available data do not permit association of the mint Ve-like sequences with resistant or susceptible phenotypes.

The isolation of candidate resistance genes such as RGAs provides a useful foundation for further study of the genetics and genomics of plant disease resistance in mint. This study also describes the development of novel degenerate primers and their use in the isolation of potential Ve homologs in mint. The present work is the first report of R gene-like sequences in Mentha, and in the Lamiaceae, and the first to report the targeted isolation of Ve homologs outside of the Solanaceae. This work advances the development of M. longifolia as a model system for disease resistance in perennials and plants in general and constitutes a step toward identification of associations between candidate R genes and phenotypically assessed resistances against pathogens such as V. dahliae, for which clear resistance/susceptibility is not available in model plant species.

The isolation of a Ve homolog from M. longifolia would open opportunities for introduction of verticillium wilt resistance into wilt-susceptible commercial mint cultivars although the marker-assisted introgression of verticillium wilt resistance from M. longifolia into peppermint may be precluded by the sterility of the latter and by differences in ploidy. Transformation systems mediated by Agrobacterium tumefaciens Smith & Townsend are available for peppermint and other commercial mints (Niu et al., 1998). The Ve-like mint sequences reported here provide a potential tool for isolation of full-length, functional Ve alleles as candidates for introduction into wilt-susceptible commercial mint cultivars.

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