Tomato *Ve* disease resistance genes encode cell surface-like receptors

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In tomato, *Ve* is implicated in race-specific resistance to infection by *Verticillium* species causing crop disease. Characterization of the *Ve* locus involved positional cloning and isolation of two closely linked inverted genes. Expression of individual *Ve* genes in susceptible potato plants conferred resistance to an aggressive race 1 isolate of *Verticillium albo-atrum*. The deduced primary structure of *Ve*1 and *Ve*2 included a hydrophobic N-terminal signal peptide, leucine-rich repeats containing 28 or 35 potential glycosylation sites, a hydrophobic membrane-spanning domain, and a C-terminal domain with the mammalian E/DXXXLϕ or YXXϕ endocytosis signals (ϕ is an amino acid with a hydrophobic side chain). A leucine zipper-like sequence occurs in the hydrophobic N-terminal signal peptide of *Ve*1 and a Pro-Glu-Ser-Thr (PEST)-like sequence resides in the C-terminal domain of *Ve*2. These structures suggest that the *Ve* genes encode a class of cell-surface glycoproteins with receptor-mediated endocytosis-like signals and leucine zipper or PEST sequences.

verticillium wilt | *Ve*1 | *Ve*2 | *Lycopersicon esculentum* | *Solanum tuberosum*

Verticillium wilt is a common fungal disease that causes severe yield and quality losses in many crops, including alfalfa, cotton, cucurbits, eggplant, mint, potato, tomato, strawberry, and sunflower (1). Several species of *Verticillium* have been reported to cause wilt and control often has relied on the use of expensive chemical fumigants that may impact health and environment adversely. In a few cases, effective control of verticillium wilt has been reported in specific crops that exhibit race-specific resistance (2, 3).

Plant resistance to viruses, bacteria, and fungi frequently involves specific host–pathogen interactions between the products of a plant resistance gene (*R*) and corresponding avirulence gene (*Avr*) in the pathogen (4, 5). Absence of either of these entities results in a susceptible response whereby plant defenses are not elicited and infection proceeds. Several plant disease resistance genes have been cloned and assigned to one of five classes based on structural features. One class includes *R* genes that encode a cytoplasmic serine/threonine protein kinase such as *Pto* in *Pseudomonas syringae* (6). Another class includes the *P. syringae RPS*2 and *RPM*1 resistance genes of *Arabidopsis* and the tomato *Fusarium oxysporum* resistance gene *I*2, which encode cytoplasmic proteins with a leucine zipper, a nucleotide-binding site (NBS), and a C-terminal leucine-rich repeat (LRR) (7–10). A third class of cytoplasmic proteins possess LRR and NBS motifs and an N-terminal domain with homology to the mammalian Toll/interleukin-1 receptor domain. This class includes the tobacco *N* gene for resistance against tobacco mosaic virus, the flax *L*6 gene for resistance to *Melamspora lini*, and the *Arabidopsis RPP*5 resistance gene for *Peronospora parasitica* (11–13). A fourth class consists of the tomato *Cladosporium fulvum* resistance genes that have an extracellular LRR, a membrane-spanning domain, and a short cytoplasmic C terminus (14). The rice *Xa*21 resistance gene for *Xanthomonas* represents a fifth class having an extracellular LRR and an intracellular serine/threonine kinase domain (15).

In tomato (*Lycopersicon esculentum*), resistance to race 1 of *Verticillium dahliae* and other species is conferred by a single dominant *Ve* gene that was mapped to linkage group IX (16). We identified in near-isogenic tomato germplasm a codominant random-amplified polymorphic DNA (RAPD) marker within 3.2 ± 0.3 centimorgans (cM) of *Ve* (17). Sequences of the RAPD were used subsequently to develop allele-specific sequence-characterized amplified regions (SCARs) determined by high-resolution mapping to be within 0.67 ± 0.49 cM or 290 kb of *Ve* (18).

We describe in this study the positional cloning of two inverted resistance genes from the tomato *Ve* locus and demonstrate that both *Ve*1 and *Ve*2 independently confer resistance to an aggressive race 1 isolate of *V. albo-atrum* in potato. Structures within the *Ve* genes suggest they encode a class of cell-surface glycoproteins with signals for receptor-mediated endocytosis and leucine zipper or Pro-Glu-Ser-Thr (PEST) sequences.

PLANT BIOLOGY

Materials and Methods

Screening of Tomato Genomic and cDNA Libraries. Genomic clones (Fig. 1) were isolated by initially screening a λ EMBL3 library (CLONTECH) of the *V. dahliae* race 1-resistant *L. esculentum* germplasm VFN8 with allele-specific SCARs (18). Approximately three copies of the genome, represented by 2×10^5 recombinant plaques, were transferred to duplicate Hybond N^+ membranes and were probed. Rescued clones were subcloned into pBluescript SK(−) (Stratagene) and were sequenced.

A Stratagene cDNA cloning kit was used to prepare and unidirectionally clone cDNA as described by the manufacturer. Total RNA was isolated from detached leaves of greenhouse-propagated verticillium wilt-resistant *L. esculentum* cultivar Craigella, stressed in 1 mM L-serine for 48 h. Polyadenylated [poly(A)$^+$] RNA was isolated by oligo(dT)-cellulose chromatography. First-strand cDNA synthesis was primed with an oligo(dT) linker-primer that contained an *Xho*I site and was reverse transcribed by using an RNase H^- reverse transcriptase in the presence of 5-methyl-dCTP to hemimethylate the cDNA. Second-strand cDNA was prepared by using RNase H and DNA polymerase I, and the double-stranded DNA was treated with the Klenow fragment of DNA polymerase before ligation to *Eco*RI adapters. The cDNA was ligated to *Eco*RI- and *Xho*I-restricted arms of the λ Uni-ZAP XR vector. Phage were

Abbreviations: LRR, leucine-rich repeat; SCAR, sequence-characterized amplified regions; PEST, Pro-Glu-Ser-Thr.

Data deposition: The sequences reported in this paper have been deposited in the GenBank database [accession nos. AF272366 (*Ve*1 cDNA), AF272367 (*Ve*1 genomic), AF365930 (*Ve*2 cDNA), and AF365929 (*Ve*2 genomic)].

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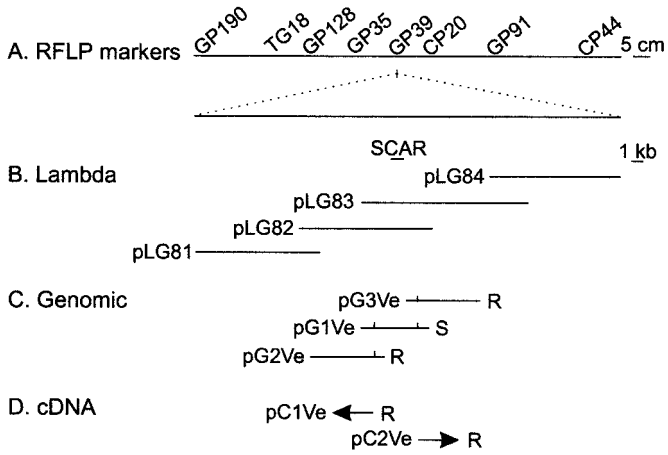

Fig. 1. Schematic genetic and physical representation of *L. esculentum* linkage group (*A*) bordering the *Ve* gene. Analysis of populations segregating for *Ve* identified closely linked codominant random-amplified polymorphic DNAs and allele-specific SCARs that map to the region of RFLP GP39. Identification of contiguous λ-genomic clones (*B*) facilitated the subcloning of genomic DNA (*C*) containing the *Ve* locus. Vertical lines indicate the location of the AUG initiation codon in the subgenomic clones. Expressed sequences were cloned into λ, and arrowheads depict the direction of transcription for the cDNAs (*D*) identified by using the genomic clone pG1Ve. Potato plants transformed with the genomic subclones pG2Ve and pG3Ve or cDNAs pC1Ve and pC2Ve exhibited *in vivo* complementation and resistance (R) when challenged with *V. albo-atrum* race 1. Potato plants transformed with the genomic pG1Ve and the binary vectors pBIN19 and pBI121 were susceptible (S) to infection.

packaged and used to infect the *recA*$^-$ *Escherichia coli* XL1-Blue MRF′. Approximately 3×10^5 recombinant plaques were transferred to Hybond N$^+$ membranes and were screened with the genomic subclone pG1Ve. Eight cDNA clones were recovered and pBluescript SK(−) phagemid with the cloned inserts were excised and recircularized.

Southern and Transcription Analyses. Genomic and cDNA clones linked to *Ve* were subcloned into the pBIN19 or pBI121 binary vectors for *Agrobacterium tumefaciens*-mediated transformation of potato (19). Stable integration of the sequences between the T-DNA borders was confirmed by Southern analysis (20). Isolated genomic DNA was restricted, separated by gel electrophoresis, transferred to Hybond N$^+$ membranes (Amersham Pharmacia), and hybridized with pC1Ve as recommended by the membrane manufacturer. Expression of *Ve* in potato was determined by reverse transcription and amplification with the PCR. Total RNA was isolated from fresh plant material by using TRIZOL (Life Technologies, Rockville, MD) and 2.5 μg of RNA treated with RNase-free DNase I (Life Technologies) before cDNA synthesis with the ThermoScript RT-PCR system (Life Technologies). Reverse transcription was performed at 68°C (*Ve*1) or 58°C (*Ve*2) with 500 nM of gene-specific primer 5′-CTGGTTTCAACTCTGAAGTATC-3′ (*Ve*1) or 5′-ATTTGCTGCCCCTACTATGTATCC-3′ (*Ve*2), complementary to 3′ untranslated sequences. Subsequent PCR was performed with an annealing temperature of 68°C (*Ve*1) or 66°C (*Ve*2) in a 50-μl volume containing 10% (vol/vol) of cDNA reaction, 0.2 μM cDNA primer, and 5′-TAACAGTCTTGTTGATCGTTTCCC-3′ (*Ve*1) or 5′-TGAATTGTAAGTTGTTGGAGGTCC-3′ (*Ve*2) primers specific to 5′ exon sequences.

Resistance Complementation Assays. Plants propagated in the greenhouse were inoculated with aggressive isolates of *V. albo-atrum* race 1 (18) or *Phytophthora infestans* US8 mating type A2 (21). Disease reactions were obtained by challenging a minimum of 10 plants from at least three independent lines of transgenic potato plants for each construct. Plants ≈15 cm in height were inoculated with *V. albo-atrum* by removing the lower roots before submerging the remaining roots for 10 min in a 5×10^7 conidia per milliliter suspension. Alternatively, leaves and stems were immersed in a 5×10^4 sporangia and zoospore suspension of *P. infestans*. Plants were rated 3 weeks postinoculation as either susceptible to the pathogen, as indicated by chlorosis and necrosis of the leaves and stunting of the plant, or as resistant if there were no disease symptoms and appearance was similar to the uninoculated plants.

Fig. 2. Genetic complementation in potato plants transformed with *Ve*. Disease symptoms were recorded 3 weeks after plants containing pG2Ve::pBIN19 (left) (resistant) or pBIN19 (right) (susceptible) were inoculated with *V. albo-atrum* race 1. Similar disease resistance was observed in plants transformed with pG3Ve::pBIN19, pC1Ve::pBI121, and pC2Ve::pBI121.

Sequence Analysis. Genomic DNA and cDNA sequences were determined with a Sequitherm Long-Read Cycle Sequencing kit (Epicentre Technologies, Madison, WI) and an ABI 377 automated sequencer (PE Biosystems, Foster City, CA) by using primers derived from the genomic sequences and the polylinker cloning site of the vectors. Various versions of the BLAST algorithm (22) were used to search DNA and protein databases for similarity. Motifs were identified with the PCGENE (IntelliGenetics) program version 6.8.

Results

Positional Cloning of *Ve* and Genomic Complementation in Transgenic Potato Plants. To proceed with map-based cloning of *Ve*, we used the SCAR sequences as hybridization probes to identify λ clones that possessed contiguous, overlapping inserts of genomic DNA (Fig. 1) from resistant *L. esculentum* VFN8 germplasm. Identification of *Ve* involved *in vivo* functional complementation within the potato (*Solanum tuberosum* ssp. *tuberosum*) cultivar Désirée, which is highly susceptible to verticillium wilt. *In vivo* complementation and specificity were observed initially in potato plants transformed with the 4-kb genomic sequences of λ-subclone pG2Ve and subsequently pG3Ve (Fig. 1). These plants exhibited delayed and reduced disease symptoms after inoculation with *V. albo-atrum* race 1 (Fig. 2) but no resistance to *P. infestans* (Table 1). All untransformed plants and control plants transformed with the binary vector pBIN19 or pG1Ve displayed wilt, chlorosis, and necrosis within a few weeks of *V. albo-atrum* race 1 inoculation.

Identification of Expressed Sequences and cDNA Complementation in Transgenic Potato Plants. To identify expressed sequences and the *Ve* locus, the λ subclones were sequenced and pG1Ve was used to probe an *L. esculentum* cDNA library of the verticillium

Table 1. Disease incidence in potato and tomato genomes challenged with aggressive isolates of *V. albo-atrum* race 1 and *P. infestans* A2 US8

	V. albo-atrum race 1		*P. infestans* A2 US8	
Line*	R	S	R	S
Potato				
pG1Ve	0	31	0	8
pG2Ve	48	0	0	8
pG3Ve	83	0	0	8
pC1Ve	30	0	0	8
pC2Ve	78	0	0	8
pBIN	0	48	0	8
pBI122	0	48	0	8
Désirée	0	56	0	8
Tomato				
Ailsa Craig	0	32	0	32
Craigella	32	0	0	32

Plants were rated 3 weeks postinoculation as resistant (R, no disease symptoms) or susceptible (S, advanced necrosis). Complementation was observed only in plants challenged with *V. albo-atrum* race 1 expressing full-length genomic DNA or cDNA of *Ve*1 (pG2Ve and pC1Ve) or *Ve*2 (pG3Ve and pC2Ve).

*Transformants pG1Ve, pG2Ve, and pG3Ve contain genomic DNA with the intergenic region and N terminus of *Ve*1 and *Ve*2, a full-length clone of *Ve*1, or a full-length clone of *Ve*2, respectively. Plants transformed with pC1Ve (*Ve*1) and pC2Ve (*Ve*2) express full-length cDNA clones under the transcriptional control of the cauliflower mosaic virus 35S promoter.

wilt-resistant tomato variety Craigella. Genomic sequences confirmed that pG1Ve possessed the SCAR sequence linked to the resistant *Ve* allele and revealed inverted terminal ORFs in pG1Ve homologous to the N-terminal domain of plant and animal receptors that possess LRRs. The cDNA clones, pC1Ve and pC2Ve, corresponding to the ORF sequences detected in the genomic subclone pG1Ve, were isolated and designated *Ve*1 and *Ve*2 (Fig. 1). To confirm complementation observed with the genomic clones, the cDNA of pC1Ve and pC2Ve was cloned into the binary vector pBI121 in a sense orientation under transcriptional control of the cauliflower mosaic virus 35S promoter for plant transformation. All plants expressing pC1Ve and pC2Ve cDNA were resistant to *V. albo-atrum* race 1 but not *P. infestans*, whereas untransformed germplasm and plants transformed with the vector alone were susceptible to both pathogens (Table 1).

Deduced Primary Structure of *Ve*1 and *Ve*2. Sequence analysis of the isolated cDNA and corresponding genomic clones did not detect any introns within the *Ve* ORFs. However, an amino acid identity of 84% and several structural domains were observed within the deduced *Ve* proteins (Fig. 3). A hydrophobic N terminus in the *Ve* proteins (domain A), indicative of a signal peptide that may target the protein to the cytoplasmic membrane (23), contains a leucine zipper-like motif with four contiguous amphipathic heptad repeats in *Ve*1. Domain A precedes an LRR with 38 imperfect copies of a 24-aa consensus [XXIXNLXXLXXLX-LSXNXLSGXIP (domain B)] that is often associated with protein–protein interactions and ligand binding. The presence of a glycine within the consensus sequence is consistent with that of extracytoplasmic proteins, a location that would facilitate the recognition of an extracellular pathogen ligand (14, 15). Within the predicted LRR region, 28 or 35 sequences matching the N-glycosylation consensus sequence NX(S/T) were observed in *Ve*1 and *Ve*2, respectively (Fig. 3).

As frequently observed with membrane-spanning proteins, a hydrophobic sequence with a predicted α-helix secondary structure (domain D) is flanked by a negatively charged extracytoplasmic domain C and a positively charged cytoplasmic domain E. Each cytoplasmic domain possesses the dileucine E/DXXXLϕ or tyrosine YXXϕ signal sequences, where ϕ is an amino acid with a hydrophobic side chain, that stimulate receptor-mediated endocytosis and degradation of mammalian cell-surface receptors (24). In *Ve*2, the C terminus also contains a PEST-like sequence found in proteins with cytoplasmic half-lives of only a few hours (25) and concludes with the residues KKF, similar to the KKX motif that signals endoplasmic reticulum retention in mammalian and plant cells (26, 27).

Discussion

Verticillium wilt resistance has been incorporated into most commercial tomato varieties and has proven to be very durable. We report in this study the positional cloning of the verticillium wilt *Ve* resistance genes from tomato. Identification of two closely linked inverted genes independently conferring resistance to the same pathogen was unexpected. The only other example of two closely linked functional disease resistance genes is the *Cf*2 locus, which consists of direct repeats differing by only three nucleotides (28). Like *Cf*2, the *Ve* genes likely resulted from relatively recent gene duplication and homologous recombination events that are believed to contribute to *R* gene evolution. It is possible that the *Ve* receptors recognize different ligands that would require a pathogen to possess at least two virulence products before inciting disease.

Homology to the *Ve* genes was observed in genes encoding several plant proteins with LRRs, including disease resistance genes that probably produce cytoplasmic proteins (6–13) and the *Xa* and *Cf* genes (14, 15) reported to encode proteins with an extracytoplasmic domain that interacts with an extracellular ligand. Unlike *Xa*21, the *Ve* genes do not include a protein kinase and are unique, because they are the only disease-resistant receptors containing endocytosis-like signals and leucine zipper or PEST sequences. Leucine zippers have been reported in the cytoplasmic class of *Arabidopsis* resistance genes *RPS*2 and *RPM*1 for *P. syringae* and can facilitate dimerization of proteins through the formation of coiled-coil structures (29). PEST sequences are often involved in ubiquitinization, internalization, and degradation of proteins (30). Discovery of a leucine zipper, PEST, and endocytosis signals in the *Ve* receptors expands the available motifs and complexity of plant cell receptors.

All eukaryotic cells exhibit receptor-mediated endocytosis as a mechanism to communicate or respond to external stimuli. In mammalian cells, ligand-dependent (e.g., insulin and cytokine CD4 receptors) or constitutive (e.g., transferrin and low density lipoprotein receptors) endocytosis is stimulated by tyrosine or dileucine motifs located within the cytoplasmic C terminus, concentrating cell-surface receptors into clathrin-coated pits that are internalized and degraded in the lysosome (31). In plant cells, clathrin-coated pits and other indirect evidence of receptor-mediated endocytosis has been observed (32). Identification of the EKCLLW and YCVF sequences in the short cytoplasmic domains of two homologous but independent protein sequences indicates that plant and mammalian cell-surface receptors may share similar endocytosis signals. In *Ve*, receptor-mediated endocytosis could provide a mechanism through which cells selectively capture ligands and remove signaling receptors from their surfaces, thereby actively responding to changing disease pressures.

Cytoplasmic signaling by *Ve* may be analogous to that of the erythropoietin cytokine receptor. Preformed dimers on the cell surface facilitate transmission of a ligand-induced conformational change from the extracytoplasmic to the cytoplasmic domain and subsequent signal transduction (33). The cytoplasmic domain interacts with kinases that link ligand binding to tyrosine phosphorylation of various signaling proteins and transcription-activation factors. A similar model has been proposed

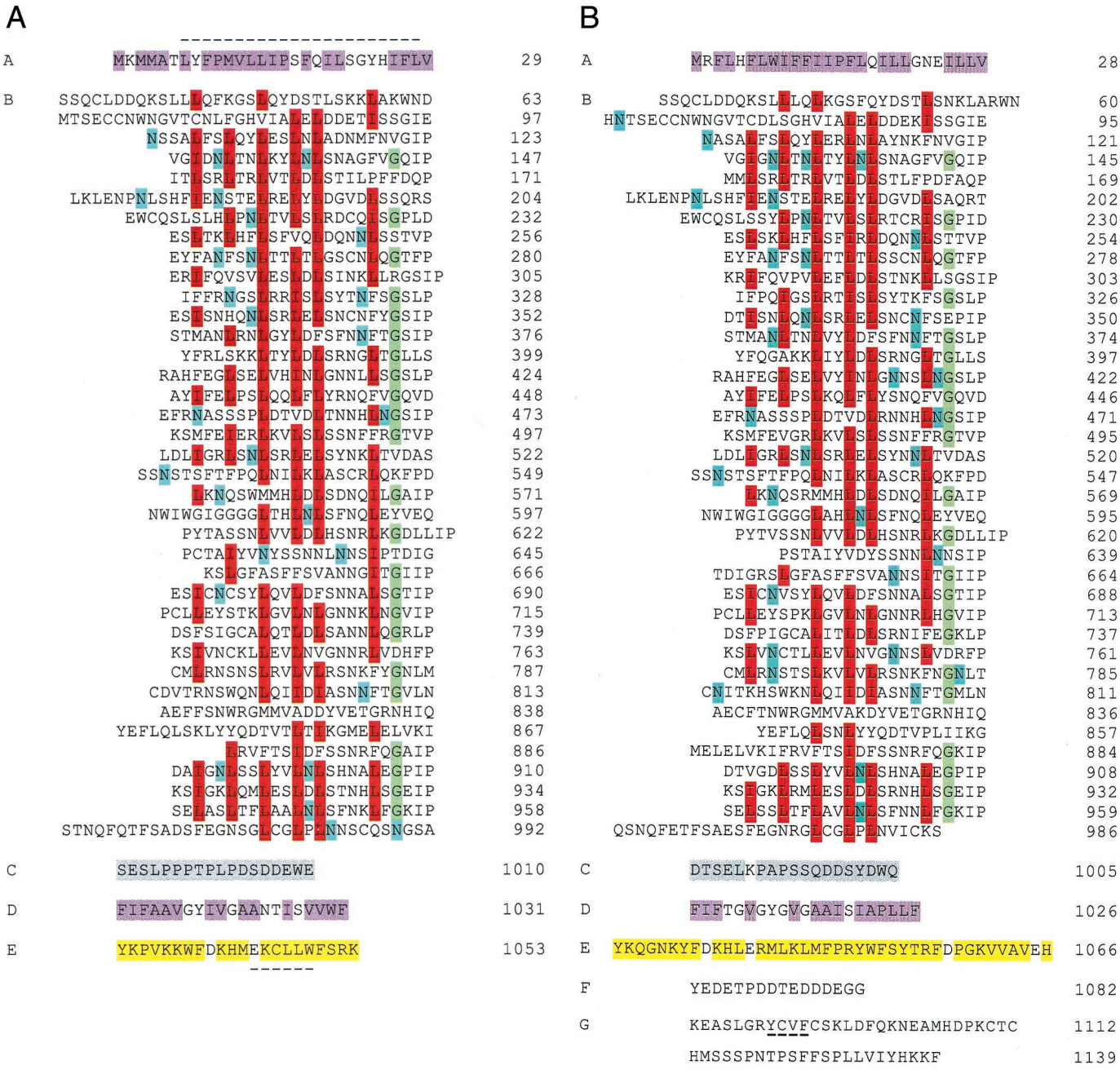

Fig. 3. Primary structure of the *Ve*1 (*A*) and *Ve*2 (*B*) proteins deduced from cDNA sequence. The polypeptides have been divided into domains A–G as described in the text. A dashed line occurs above the putative N-terminal leucine zipper in domain A of *Ve*1 and below the endocytosis signals in domain E of *Ve*1 and domain G of *Ve*2. Highlighted are the hydrophobic amino acids (purple) of the putative signal peptide domain A and membrane-associated domain D; conserved L/I (red), G (green), and potential N-glycosylation sites (blue) within the LRR domain B; neutral and acidic amino acids (gray) of domain C; and neutral and basic amino acids (yellow) of domain E. The PEST sequence of *Ve*2 is shown in domain F.

for the kinase encoded by the *Pto* resistance gene that lacks a receptor domain (6) and *Cf* extracytoplasmic receptors that lack a kinase (14, 28). Alternatively, receptor-mediated endocytosis may allow the extracellular domains and ligands of the *Ve* proteins to directly stimulate signal transduction.

Resistance to different pathogen species is contrary to the traditional view of a highly specific interaction with race-defining *R* genes. Our results demonstrate that, although the tomato *Ve* genes have the specificity to distinguish races 1 and 2 of *V. dahliae,* the genes also possess the capacity to recognize another *Verticillium* species in a different host. This pleiotropic resistance resembles that observed with the *Mi* gene, which confers resistance to nematodes and aphids (34, 35) and shares the ability of *R* genes to retain biological activity in other plant genera (8, 36). Several *Verticillium* species infect many agricultural plants and this pleiotropic, host-independent complementation should be of considerable value.

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