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The *I2C* Family from the Wilt Disease Resistance Locus *I2* Belongs to the Nucleotide Binding, Leucine-Rich Repeat Superfamily of Plant Resistance Genes

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Characterization of plant resistance genes is an important step in understanding plant defense mechanisms. *Fusarium oxysporum* f sp *lycopersici* is the causal agent of a vascular wilt disease in tomato. Genes conferring resistance to plant vascular diseases have yet to be described molecularly. Members of a new multigene family, complex *I2C*, were isolated by map-based cloning from the *I2 F. o. lycopersici* race 2 resistance locus. The genes show structural similarity to the group of recently isolated resistance genes that contain a nucleotide binding motif and leucine-rich repeats. Importantly, the presence of *I2C* antisense transgenes abrogated race 2 but not race 1 resistance in otherwise normal plants. Expression of the complete sense *I2C-1* transgene conferred significant but partial resistance to *F. o. lycopersici* race 2. All members of the *I2C* gene family have been mapped genetically and are dispersed on three different chromosomes. Some of the *I2C* members cosegregate with other tomato resistance loci. Comparison within the leucine-rich repeat region of *I2C* gene family members shows that they differ from each other mainly by insertions or deletions.

INTRODUCTION

Resistance to pathogens is thought to involve a specific recognition between a resistant plant and the pathogen. This interaction triggers a set of responses that act to confine the pathogen. The specificity of this process is often determined by the product of a plant resistance (*R*) gene and a cognate pathogen avirulence gene (Flor, 1971). The characterization of *R* genes is of major importance in elucidating the initiation of the cascade of events that leads to defense responses in important crops.

Recently, several *R* genes have been cloned by positional cloning or transposon tagging (Johal and Briggs, 1992; Dangl, 1995; Staskawicz et al., 1995; Boyes et al., 1996). Despite their origin from different plant species and their divergent specificity to viral, fungal, or bacterial pathogens, a group of these *R* genes, designated the nucleotide binding, leucine-rich repeat (*NB-LRR*) group, shares several features. These *R* genes are all involved in resistance processes characterized by a hypersensitive response (HR). Structurally, a nucleotide binding domain (P loop) and additional motifs of unknown function are conserved near their N-terminal regions. A region of LRRs of variable length and content is present at their C terminus and may play a role in protein–protein interactions (Kobe and Deisenhofer, 1995a). Several *R* genes have been shown to belong to large, clustered gene families (Jones et al., 1994; Whitham et al., 1994; Lawrence et al., 1995; Dixon et al., 1996). However, the detailed genomic distribution of these multigene families is as yet not known.

The soil-borne fungus *Fusarium oxysporum* f sp *lycopersici* is the causal agent of severe wilt diseases in tomatoes. The fungus penetrates the vascular system of roots mainly through wounds and proceeds through the vascular system, leading to its functional collapse, systemic wilting, and often death of the plant. In resistant plants, the fungus is confined to the lower part of the roots, and additional symptoms do not develop (Beckman, 1987). Several mechanisms that differ in nature from the HR were suggested to be involved in this resistance. They include the production of inhibitory secondary metabolites and the formation of structural barriers in vascular sieve elements that are caused by gelation, callose deposition, and membranal outgrowths from the vascular parenchyma cells, termed tyloses. Most of these resistance processes are detectable in compatible interactions as well, albeit to a lesser extent, and resistance may involve the more rapid and synchronized appearance of the defense mechanisms (Beckman, 1987).

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Three races of *F. o. lycopersici* and their cognate *R* genes have been identified in tomato. The classification of different *F. o. lycopersici* isolates into races based on virulence is independent of their general genetic resemblance, as established by both restriction fragment length polymorphism (RFLP) analysis and distribution into vegetative compatibility groups (Elias et al., 1993). In tomato, the *I* locus, introgressed from *Lycopersicon pimpinellifolium*, confers resistance to *F. o. lycopersici* race 1 and is located on the short arm of chromosome 11 between RFLP markers *TG523* and *CP58* (Y. Eshed and N. Ori, unpublished data). The *I3* locus from chromosome 7 of *L. pennellii* confers resistance to races 1, 2, and 3 of *F. o. lycopersici* (Bournival et al., 1990; Sarfatti et al., 1991). The *I2* locus, introgressed from *L. pimpinellifolium*, confers resistance to race 2 of the pathogen. It was mapped to the long arm of chromosome 11 (Segal et al., 1992). Here, we present high-resolution genetic and physical mapping of the *I2* region by using a large F_2 population. Our results show complete cosegregation between *I2* and a new gene complex, *I2C*, that shows structural features similar to those of the *NB-LRR* type of *R* genes. *I2C* antisense transgenes specifically abrogate *F. o. lycopersici* race 2 resistance, and a member of this family, *I2C-1*, confers significant but not complete *F. o. lycopersici* race 2 resistance to otherwise susceptible plants.

RESULTS

High-Resolution Mapping of *I2*

The *I2 F. oxysporum R* gene was previously mapped to chromosome 11 between RFLP markers *TG105A* and *TG36* and is 0.4 centimorgans (cM) from *TG105A* (Figure 1A; Segal et al., 1992). To obtain higher resolution mapping of *I2*, we generated new markers in the region of *TG105A* by chromosome walking from *TG105A* on λ clones and by subcloning a 350-kb yeast artificial chromosome (YAC) clone, YAC340-G3, that hybridized with *TG105A*. Pulsed-field gel electrophoresis of YAC340-G3 was used to physically position genetically informative markers, as shown in Figure 1C. To localize the position of *I2* relative to the new markers, a segregating population of 1600 plants (F_2 and F_3; see Methods) was screened for recombination events between *TG105A* and *TG36*, and 57 recombination events were detected. The recombinant plants were then tested for *F. o. lycopersici* race 2 resistance with additional RFLP markers. According to the resulting map (Figure 1B), *I2* maps to the *SL8D* locus, identified by the multicopy RFLP marker *SL8*, which represents the edge of YAC340-G3 (Figure 1C), lies genetically between markers *6-16* and *TG36*, and is 0.23 cM from *6-16* and 1.3 cM from *TG36*.

In previous studies, we used recombinant inbred (RI) lines to map *I2* (Ori et al., 1994). Due to inconsistencies in the linearity of markers between RI lines and all of the F_2 populations described here, the use of RI lines was discontinued. Interestingly, however, *SL8D* completely cosegregated with *I2* in the RI lines as well (data not shown). Compared with flanking markers, the *SL8* marker showed a remarkably high rate of polymorphism between *F. o. lycopersici* race 2–resistant and –susceptible lines (Figure 2).

SL8 Is a Member of a Gene Family Cosegregating with *I2*

The complete genetic cosegregation of *SL8D* with the *I2* resistance gene prompted us to further characterize the multicopy marker *SL8*. Sequence analysis revealed that *SL8* contains an open reading frame with similarity to a group of recently isolated *R* genes (see below). This suggested that *SL8* is a part of a gene belonging to a family that includes the *I2 R* gene. This gene carrying the *SL8* probe at the 3′ end was therefore designated *I2C-1* (*I2* complex 1).

Our next step was to characterize further the different *SL8* copies as RFLP markers, draw criteria to distinguish between them, and analyze their genomic distribution. A comparison of the *SL8* RFLP patterns of resistant- and susceptible-type lines obtained with the restriction enzyme TaqI is shown in Figure 2. Resistant-type bands, consistent among all tested lines, were designated TR1 to TR8. The rest of the bands were either nonpolymorphic or polymorphic between the different pedigrees of susceptible lines. Only one recombinant plant, which was susceptible to *F. o. lycopersici* race 2,

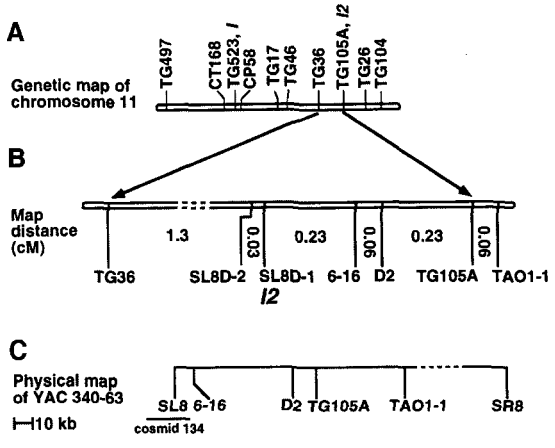

Figure 1. Genetic and Physical Map of the *I2* Region.

(A) Abridged genetic linkage map of chromosome 11, adapted from Eshed et al. (1995). The *I* and *I2 F. oxysporum R* loci were positioned according to Y. Eshed (unpublished data) and Segal et al. (1992), respectively.

(B) Results of high-resolution mapping of the genetic region spanning RFLP markers *TAO1-1* through *TG36*, as determined from the analysis of 1600 F_2 and F_3 individuals.

(C) Physical map of YAC340-G3, with the relevant markers indicated. The total length of YAC340-G3 is 350 kb.

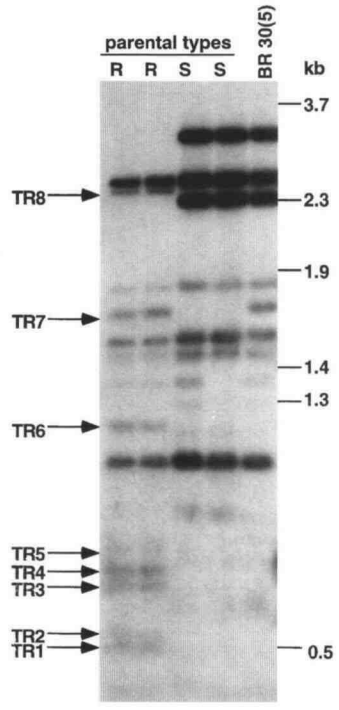

Figure 2. Gel Blot Analysis of Genomic Tomato DNA from Resistant and Susceptible Parental Types and of the Recombinant F_2 Plant BR 30(5).

DNA samples were digested with TaqI, and the blot was hybridized with the *SL8* probe. R and S indicate *F. o. lycopersici* race 2–resistant and race 2–susceptible individuals, respectively. R lanes represent parental types that are nonrecombinant resistant-type F_2 individuals from the F_2 population initiated from Br5577; S lanes represent parental lines that are the susceptible tomato inbreds *L. esculentum* cvs M82 and S-365. BR 30(5) is the single recombinant identified within the *SL8D* cluster from the entire F_2 population. TR1 to TR8 indicate resistant-type polymorphic bands. Molecular length markers are indicated at right in kilobases.

was recovered from the entire F_2 population (BR 30(5) in Figure 2). This separates the *SL8D* locus into two distinct loci, *SL8D-1* and *SL8D-2* (Figure 1B). The recombinant individual BR 30(5) contains one resistant-type TaqI band, TR7, but lacks the others (TR1 to TR6 and TR8). Thus, bands TR1 to TR6 and TR8 appear to cosegregate completely with each other and with *I2* and are therefore candidates for the *R* gene. The possibility that nonpolymorphic bands represent the *R* gene is unlikely because most nonpolymorphic bands map to different loci (see below).

Genomic Distribution of *I2C*

Because only a subset of the *SL8* copies showed polymorphisms between the parents of the F_2 populations used for mapping, a series of 50 introgression lines (ILs) was incorporated to map all of the *SL8* fragments. Each line contains a single chromosome segment from *L. pennellii* introgressed into an *L. esculentum* background (Eshed and Zamir, 1995). Figure 3A illustrates the introgression of genomic segments from chromosomes 8, 9, and 11 in the ILs. These lines proved to be relevant for *SL8* mapping. All *SL8* copies from *L. esculentum* and *L. pennellii* appeared to be polymorphic (Figure 3B, lanes 1 and 2). This feature facilitated their mapping. DNA digests of *SL8* from the ILs were compared with those of *L. esculentum* and *L. pennellii* by using DNA gel blot hybridization analysis (Figure 3B). *L. pennellii* bands that are contained in each IL, as well as their allelic *L. esculentum* bands that are absent from these lines, represent *SL8* copies that originated from the region of introgression in the respective line. One *SL8* copy was localized to the short arm of chromosome 8 (*SL8A*), because one *L. pennellii*–type polymorphism is present and one *L. esculentum*–type polymorphism is absent from IL 8-1. Similarly, another *S18* copy was localized near the centromere of chromosome 9 (*SL8B*; Figures 3A and 3B). More accurate mapping of these two copies was obtained by using lines containing shorter introgressed segments of the region. These lines were derived by selection of recombinants from the F_2 progeny of the original IL crossed back to *L. esculentum* cv M82, as illustrated in Figure 3A.

The remaining *SL8* fragments mapped to the long arm of chromosome 11. Two of the lines, IL 11-3 and IL 11-4, contain *L. pennellii* segments that map to the long arm of chromosome 11 (Figure 3A). By comparing these two lines, three genetically distinct groups of *SL8* family members could be identified on chromosome 11. The first (*SL8C*) maps to the region exclusively marked by introgression in IL 11-3, the second (*SL8D*) to the region of overlap between IL 11-3 and IL 11-4, and the third (*SL8E*) to the region exclusively marked by introgression in IL 11-4. As previously established, the *I2* resistance gene maps to the region of overlap designated *SL8D.* Higher resolution mapping of the chromosome 11–based *SL8* loci was accomplished by an F_2 population of 150 plants generated from an initial cross between *L. esculentum* and an IL containing the long arm of chromosome 11 (line 11; Eshed et al., 1992). Analysis of the F_2 population corroborated the division of *SL8* markers into clusters. *SL8C* and *SL8D* cosegregated completely with the RFLP markers *TG546* and *6-16*, respectively, and *SL8E* mapped between markers *TG26* and *TG105* at 0.25 cM from *TG26* (Figures 1 and 3).

Interestingly, the *SL8B* locus from chromosome 9 cosegregates with markers completely linked to the *Tm-2a* tobacco mosaic virus *R* gene (Young et al., 1988) and within 5 cM of the *Frl F. o. radicis lycopersici R* gene (Laterrot and Moretti, 1995). The *SL8C* cluster maps within 10 cM of the *Sm Stemphylium* spp *R* gene (Behare et al., 1991). However, none of the *SL8* loci cosegregates with the *I3 F. o. lycopersici R* locus situated on chromosome 7, conferring resistance to races 1, 2, and 3 of *F. o. lycopersici*, or with the *I F. o.*

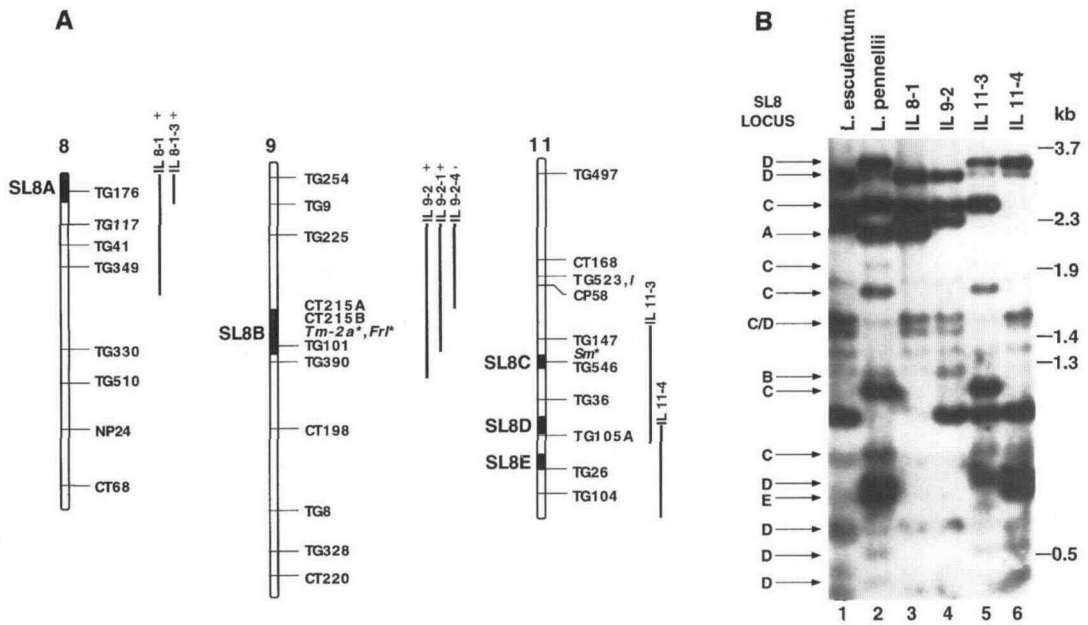

Figure 3. Distribution of *SL8* Homologs in the Tomato Genome.

(A) Linkage maps of chromosomes 8, 9, and 11 showing mapped positions of the *SL8* loci. The linkage maps were adapted from Eshed et al. (1995). The relevant regions of introgression of the ILs are shown to the right of each tomato linkage map (solid lines). The (+) and (−) indicate the presence and absence, respectively, of an *SL8* copy in the respective IL. Asterisks indicate approximate map positions of the relevant disease *R* genes, and the blackened regions indicate the mapping position of *SL8* homologs.

(B) Gel blot of TaqI-digested DNA of representative ILs. *L. pennellii*–specific fragments in the blot are designated by A to E, according to their genomic location, as indicated in **(A)**. Numbers at right indicate molecular length markers in kilobases.

lycopersici race 1 *R* locus present on the short arm of chromosome 11.

I2C Genes Share Structural Similarity with a Family of Plant *R* Genes

Candidate members of the *I2C* gene family were isolated from genomic libraries of *F. o. lycopersici* race 2–resistant tomatoes by using *SL8* as a probe and were examined for *SL8D* locus resistant-type polymorphic bands. Cosmid clone I2C-134 contains the *I2C-1* gene, which includes in its 3′ region the *SL8* marker (nucleotides 2837 to 4014; Figure 1C) and the polymorphic bands TR1 and TR5. I2C-134 also exhibits resistant-type polymorphic bands after digestion with other endonucleases, such as HindIII, DraI, and EcoRI (data not shown). Cosmid clone I2C-150 contains another gene, *I2C-2*, represented by the two polymorphic bands TR4 and TR7. The presence of both TR4- and TR7-type polymorphisms in I2C-150 could place the *I2C-2* gene to the point of recombination in BR 30(5) or arise by comigration of bands of different origin.

One continuous open reading frame was identified in both *I2C-1* and *I2C-2* (Figure 4). The C-terminal region of *I2C-1* and *I2C-2* is leucine rich and can be arranged as repeats (*I2C-1*; Figure 5A). A comparison of *I2C-1* to the *NB-LRR*–containing group of recently isolated intracellular plant *R* genes is shown in Figure 6. Although overall homology is low, an intriguing structural similarity suggests that I2C genes belong to the *NB-LRR*–containing type of *R* genes. All *NB-LRR* genes exhibit an N-terminal region of variable length and low homology, a central conserved region of comparable length, and a C-terminal LRR region (Figure 6A). The central conserved region of *I2C* and *NB-LRR*–type genes includes a putative nucleotide binding site and additional conserved regions of homology of an unknown function. The conserved stretches without gaps were grouped into seven motifs (Figure 6B, I to VII). Examination of motifs I, II, IV, and VI suggests a further subdivision into two groups, the first containing *I2C*, *RPM1*, *Prf*, and *RPS2* and the second containing *N* and *L6*.

The C-terminal region of *I2C-1* can be arranged as 27 loosely fitting repeats (Figure 5A), which is similar in general framework to other LRR-containing genes (Figure 5B). Best

overall homology of the *I2C-1* C terminus was found with the LRR region of the *T-LR* VSG expression site–associated gene from *Trypanosoma brucei* (Smiley et al., 1990; Ross et al., 1991). This protein shares 52% similarity and 25% identity with the 3′ part of *I2C-1*, and the consensus of its LRR is included in Figure 5B. The general consensus sequence LXX-LXαXX(C/N/X)X(X)α, where α designates an aliphatic amino acid and X any amino acid, followed by a region of varying length rich in P and L residues, can describe most *NB-LRR* genes. The conservation of the repeat format is considerably lower in the *NB-LRR* group than that found in other

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I2C-1 MEIGLAIGGAFLSSALNVLFDRLAPNGDLLNMFRKHTDDVELFEKLGDILLSLQIVLSDA 60
I2C-2       V                              K H K LK  KMT RGI        60

I2C-1 ENKKASNQFVSQWLHKLQTAVDAAENLIEQVNYEALRLKVETSNQQVSDL.NLCLSDDFF 119
I2C-2    Q   PS RD  NE RD   S      E          GQH NF ETS QQV  E   120

I2C-1 LNIKKKLEDTIKKLEVLEKQIGRLGLKEHFISTKQETRTPSTSLVDDSGIFGRKNEIENL 179
I2C-2     D      ET KD QE   L     Y D   L          I EPD    QS   D 180

I2C-1 VGRLLSMDTKRKNLAVVPIVGMGGMGKTTLAKAVYNDERVQKHFGLTAWFCVSEAYDAFR 239
I2C-2 ID    EGASG   T          L                 S KN  D K          N    240

I2C-1 ITKGLLQEIGSTDLKADDNLNQLQVKLKADDNLNQLQVKLKEKLNGKRFLVVLDDVWNDN 299
I2C-2           I  .............V              R KE K  I          286

I2C-1 YPEWDDLRNLFLQGDIGSKIIVTTRKESVALMMDSGAIYMGILSSEDSWALFKRHSLEHK 359
I2C-2  N   E   V V              D      GNEQ S  N  T A  S  Q  AF NM 346

I2C-1 DPKEHPEFEEVGKQIADKCKGLPLALKALAGMLRSKSEVDEWRNILRSEIWELPSCSNGI 419
I2C-2   MG S L    R   A           T           E  KC          R..D D  404

I2C-1 LPALMLSYNDLPAHLKQCFAYCAIYPKDYQFRKEQVIHLWIANGLVHQFHS.....GNQY 474
I2C-2                 R  SF   F    P                  PVEDEIIQDL    F 464

I2C-1 FIELRSRSLFEMASEPSERDVEE.FLMHDLVNDLAQIASSNHCIRLEDNKGSHMLEQCRH 533
I2C-2  L  S       RVPN   GNIK L             L   KL      ESQ           524

I2C-1 MSYSIGQDGEFEKLKSLFKSEQLRTLLPI..DIQFHYSKKLSKRVLHNILPTLRSLRALS 591
I2C-2 L   M Y  G     TP Y L         TCSSVNYF .NP T                   583

I2C-1 LSHYQIEVLPNDLFIKLKLLRFLDLSETSITKLPDSIFVLYNLETLLLSSCEYLEELPLQ 651
I2C-2     KM E                 I R N KR     C              K.        642

I2C-1 MEKLINLRHLDISNTRRLKMPLHLSRLKSLQVLVGAKFLVGGWRMEYLGEAHNLYGSLSI 711
I2C-2                 WH                          V   D    Q        V 702

I2C-1 LELENVVDRREAVKAKMREKNHVEQLSLEWSESISADNSQTERDILDELRPHKNIKAVEI 771
I2C-2 VK             P                                          QE K  762

I2C-1 TGYRGTNFPNWVADPLFVKLVHLYLRNCKDCYSLPALGQLPCLEFLSIRGMHGIRVVTEE 831
I2C-2 I                 L   K S                    K   VK             822

I2C-1 FYGRLSSKKPFNSLVKLRFEDMPEWKQWHTLGIGEFPTLEKLSIKNCPELSLEIPIQFSS 891
I2C-2                C E  E    T      A                 I              882

I2C-1 LKRLD.............................ICDCKSVTSFPFSILPTTLKRIKISGC 923
I2C-2     FRVFGCPVVFYDAQVLRSQLEGMKQIEEIY R  N .                T D    941

I2C-1 PKLKLEAPVGE..MFVEYLSVIDCGCVDDISPEFLPTARQLSIENCHNVTRFLIPTATES 981
I2C-2           C MS  L EF  EE     ...          E R G     .          T 998

I2C-1 LHIRNC...EKLSMACGGAAQLTSLNIWGCKKLKCLPELLPSLKELRLTYCPEIEGELPF 1038
I2C-2        ENV                  D S                    Q  N          1058

I2C-1 NLQILDIRYCKKLVNGRKEWHLQRLTELWIKHDGSDEHIEHWELPSSIQRLFIFNLKTLS 1098
I2C-2    K Y  D                    K V Y      D        C  T  EV   I    1118

I2C-1 SQHLKSLTSLQFLRIVGNLSQFQSQGQLSSFSHLTSLQTLQIWNFLNLQSLPESALPSSL 1158
I2C-2              Y C D    PI     I                  H     S           1178

I2C-1 SHLIISNCPNLQSLPLKGMPSSLSTLSISKCPLLTPLLEFDKGEYWTEIAHIPTIQIDEE 1218
I2C-2  Q E FH          N        K L  G                  PQ        L  W 1238

I2C-1 CM 1220
I2C-2 YI 1240
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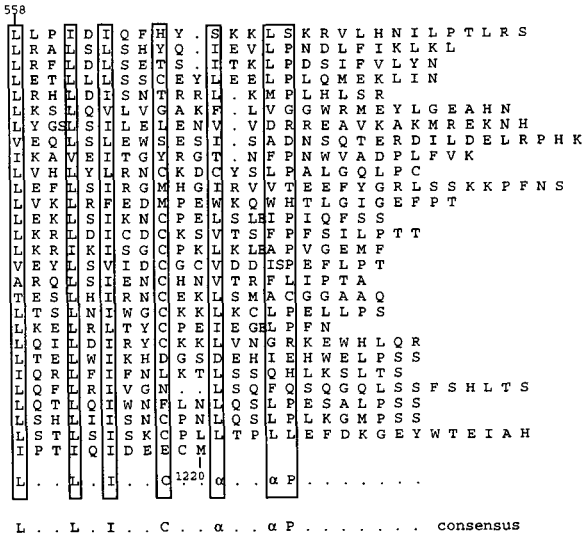
Figure 4. Amino Acid Sequence of *I2C-1* and *I2C-2* Gene Products.

The amino acid sequence of the *I2C-1* gene product is shown above, and amino acid differences in *I2C-2* are shown below. Dots represent gaps introduced to maintain alignment. Duplicated sequences are underlined. I2C-1 and I2C-2 share 87.1% similarity and 80.1% identity.

A



B

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L . . L . I . . C . . α . . α P . . . . . . .   I2-family
L . . L . L S G C . . α . . L . . L . . . . .   T-LR
L . . L . L . . . . α                          RPM1
L . . L . L . . N . . L . . I P S . L . . L .   PRF
                  C
L . . L . α . . . . . α . . α P . . α . . L .   RPS2
L . . L . L . . . . . L . . L P . . L . . L .   N
L . . L . L . . c . . α . . L                  L6
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Figure 5. Alignment of the LRRs of I2C-1.

(A) The amino acid sequence of the *I2C-1* gene product from residues 558 to 1220. The consensus sequence of the alignment is shown below.

(B) Comparison of different LRR consensus sequences: T-LR (the T-LR VSG expression site–associated leucine-rich protein from *T. brucei*; Smiley et al., 1990; Ross et al., 1991), *RPM1* (Grant, 1995), *Prf* (Salmeron et al., 1996), *RPS2* (Bent et al., 1994; Mindrinos et al., 1994), *N* (Whitham et al., 1994), and *L6* (Lawrence et al., 1995).

In **(A)** and **(B)**, dots represent any amino acid, and α represents an aliphatic residue.

known LRR-containing *R* genes, specifically those of the *Cf R* genes from tomato and the *Xa21 R* gene of maize (Jones et al., 1994; Song et al., 1995; Dixon et al., 1996).

Transcribed Sequences from the *I2C* Gene Family Contain Insertions and Frameshifts

To compare the LRR region of various resistant-type members of the *I2C* family, we sequenced two partial cDNA clones from the family, designated *I2C-3* and *I2C-4*, of 1200 and 1600 bp, respectively. One of the cDNA clones, *I2C-4*,

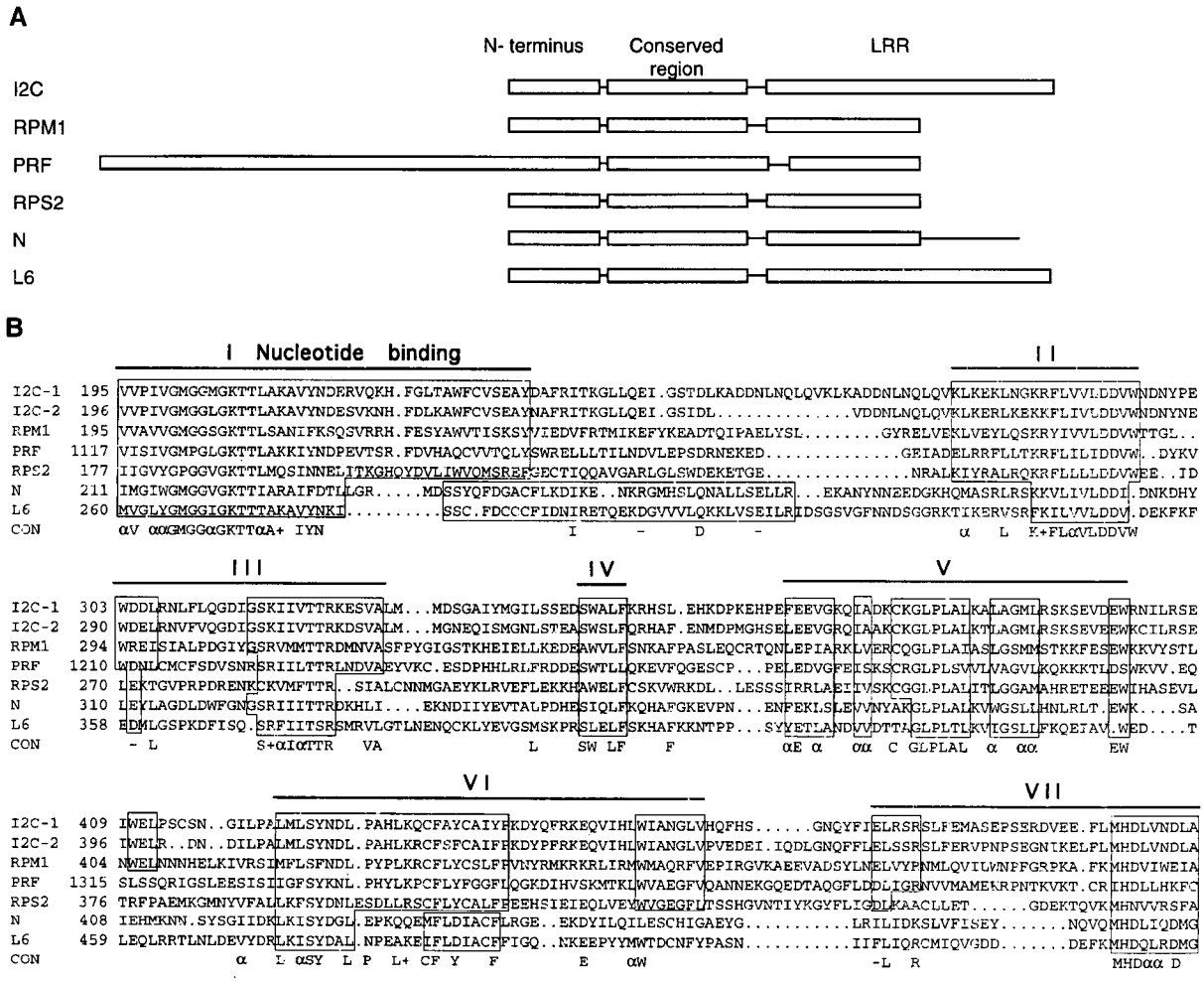

Figure 6. Structural Comparison of *I2C-1* and Members of the *NB-LRR* Group of Plant *R* Genes.

(A) Schematic comparison of *I2C-1* and other *NB-LRR R* genes (see Figure 5). The different genes share between 48 and 54% similarity and between 21 and 28% identity with *I2C-1*.

(B) Sequence comparison of the conserved region. Boxes I to VII represent conserved motifs in which similar regions are included in the same box when either no gaps or one gap was needed for alignment. The consensus (CON) sequence is indicated for at least five similar amino acids. Dots represent gaps introduced to maintain alignment, and α represents an aliphatic residue.

contains two frameshifts and thus, if translated, would result in a truncated peptide. In Figure 7, *I2C-4* is shown as a continuous chimera of the three different open reading frames. The comparison of part of the LRR region of the two genomic and two cDNA clones is shown in Figure 7. Striking insertions or deletions, mainly in the C-terminal region, can be seen. Most insertions are shared by at least two different genes, although the combinations are different for each insertion. Close to the C terminus, the different genes contain between two and six repeats of an almost identical sequence. The largest insertions in genes *I2C-3* and *I2C-4* are made up exclusively of these repeats.

I2C Antisense Transgene Abolishes *F. o. lycopersici* Resistance in a Race-Specific Manner

The complete association of *I2C* genes from the *SL8D* locus with *I2 F. o. lycopersici* race 2 resistance suggests that one or more members of the *I2C* family is the *I2* gene. We chose a gene suppression approach to test whether this family includes the *I2* gene. This approach allowed us to use individual family members to test the *I2* gene complex. Portions of the LRR region from *I2C-1* and *I2C-3* were cloned in the antisense and sense orientations downstream of the highly expressed and constitutive cauliflower mosaic virus 35S

promoter (see Methods). The constructs were transformed into the *F. o. lycopersici* race 1– and race 2–resistant tomato line Motelle (MT). All resultant transgenic plants appeared to develop and set seed normally. Examination of inoculated T_1 plants showed that transgenic plants expressing parts of *I2C-1* and *I2C-3* in the antisense and sense orientations showed a significantly increased rate of susceptibility to race 2 of *F. o. lycopersici*, as shown in Figure 8 and Table 1. Figure 8 provides an analysis of one such transformant, MT 31-17(14) (Figure 8, MT I2C antisense). The T_0 transformant of this line shows the expected resistant-type pattern in RFLP analysis (Figure 8A). In two independent experiments, 30 T_1 seedlings were infected with *F. o. lycopersici* race 2, and 80 and 60% of the seedlings showed disease symptoms. These symptoms included wilting and a marked reduction in growth (Figure 8B). The diseased transgenic plants showed fungal colonization throughout the stem length (Figure 8C). The 1:3 ratio of resistant-to-susceptible plants in MT 31-17(14) progeny is consistent with dominant segregation of a transgene that abrogates resistance. In contrast, MT 31-17(14) T_1 seedlings showed complete resistance to infection by *F. o. lycopersici* race 1 (Figures 8B and 8C, bottom). This result indicates the race 2–specific nature of the antisense inhibition. Additional independent transformants displayed varying degrees of susceptibility rates and accordingly were divided into three groups (Table 1). Approximately one-quarter of the independent transgenic lines showed complete repression of resistance, and another one-third showed partial loss, which is consistent with the vagaries of antisense and sense gene suppression. These results indicate that a member(s) of the *I2C* gene family confers resistance to *F. o. lycopersici* race 2.

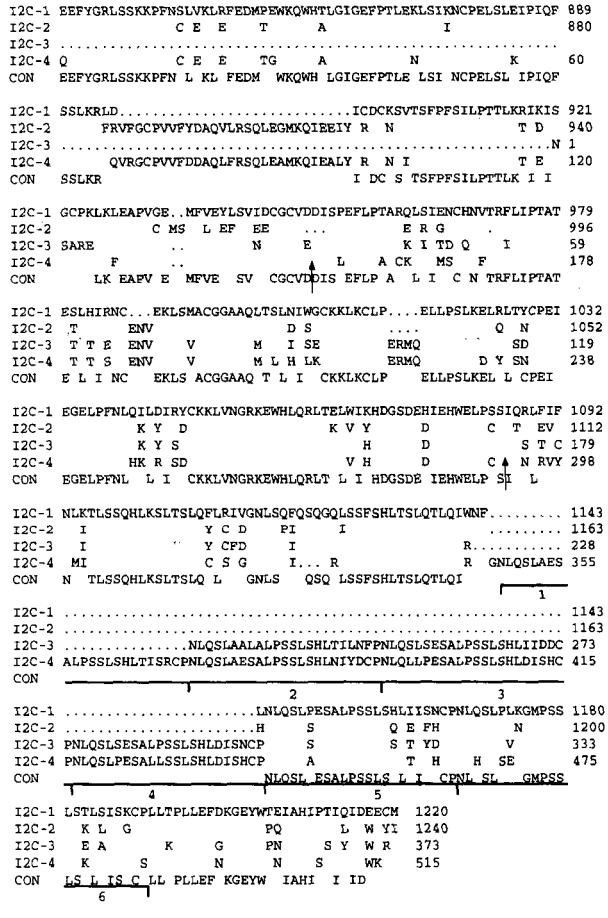

Figure 7. Comparison of the 3′ End of Four Gene Products of *I2C* Family Members.

I2C-1 and I2C-2 are as shown in Figure 4. I2C-3 and I2C-4 are the deduced sequences from partial cDNA clones that share, respectively, 87 and 84% similarity and 82 and 76% identity with the corresponding region in I2C-1. The sequence of I2C-4 is presented as a chimera between three open reading frames originally separated by one-base insertions, which caused two frameshifts. The junctions at which the separated open reading frames were combined are indicated by arrows. Consensus (CON) is shown when a residue is present in all four sequences. Numbers are from the first methionine in sequences I2C-1 and I2C-2 and from the first amino acid residue of the available sequence for I2C-3 and I2C-4. Gaps indicated by dotted lines were introduced to maintain alignment. Brackets indicate a repeat unit (1 to 6) of 23 amino acids that appears in variable copy number at the 3′ end of the cDNA clones.

I2C-1 Confers Partial Resistance to *F. o. lycopersici* Race 2

Cosmid clone I2C-134 as well as its subclones, which include only the *I2C-1* gene (see Methods), were transformed into the *F. o. lycopersici* race 2–susceptible tomato variety VF36. From a total of 15 independent transformants that set seeds, eight showed increased resistance rate in progeny tests and the rest were comparable to control VF36 plants. Generally, 0 to 25% and 85 to 100% of inoculated susceptible and resistant control plants, respectively, were symptomless when infected. In comparison, in primary infection screens of the T_1 seedlings of the eight independent *I2C-1*–containing transgenic lines, 27 to 96% were symptomless. The statistical significance of this result was established for the T_1 seedlings of three independent transformants that were infected in larger scale experiments. These results are summarized in Table 2. Chi square tests were applied to each individual experiment as well as to the pooled data. In all cases, the proportion of resistant T_1 seedlings was significantly higher than the proportion of control VF36 plants. The resistant/susceptible ratio in T_1 progeny fits a 1:3 ratio, which is consistent with the *I2C-1* conferring a recessive-type resistance. Alternatively, the gene could increase the resistance of plants in a stochastic manner so that transgenic plants are more successful in disease avoidance.

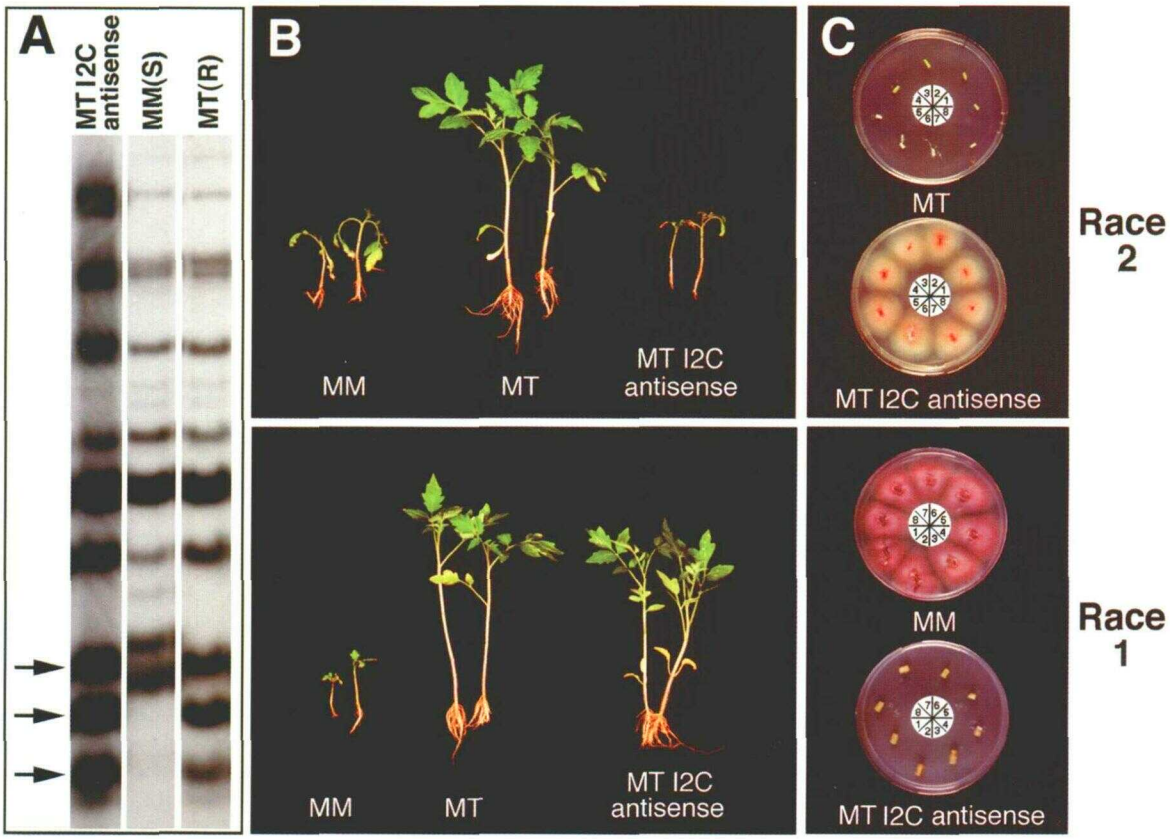

Figure 8. I2C Antisense Transgene Abrogates Resistance to *F. o. lycopersici* in a Race-Specific Manner.

L. esculentum lines shown are the near isogenic cultivars Motelle (MT; II, I2I2) and Money Maker (MM; ii, i2i2) and a transgenic plant of Motelle that contains an *I2C* antisense transgene (MT I2C antisense).

(A) Gel blot analysis of genomic tomato DNA. DNA samples were digested with DraI, and the blot was hybridized with the *SL8* probe. Arrows indicate resistant-type polymorphic bands.

(B) and **(C)** Seedlings subjected to infection with *F. o. lycopersici* race 1 or race 2, as indicated. In **(B)**, seedlings are shown 2 weeks after infection. In **(C)**, *F. o. lycopersici* colonization in stems of seedlings 2 weeks after infection is shown. Stem sections are numbered from the first true leaves (section 1) toward the roots.

DISCUSSION

Several lines of evidence strongly suggest that one or more members of the multigene *I2C* family comprise the tomato *I2* *F. o. lycopersici* race 2 *R* locus. First, the *SL8D* locus containing genes from this family completely cosegregates with the *I2* locus in a large mapping population. Second, *I2C* members contain the structural features of the *NB-LRR* group of plant *R* genes (Bent et al., 1994; Mindrinos et al., 1994; Whitham et al., 1994; Grant, 1995; Lawrence et al., 1995; Salmeron et al., 1996). Third, *I2C* antisense transgenes abrogate *F. o. lycopersici* race 2 resistance in a race-specific manner. Lastly, a member of the *I2C* family partially complements *F. o. lycopersici* race 2 susceptibility in transgenic plants.

Wide Genomic Distribution of the *I2C* Family

Using novel tomato genetic populations (Eshed and Zamir, 1995), we mapped all members of the *I2C* family. The *I2C* genes are localized in five genomic positions. Two of these are clusters of several genes that are both located on chromosome 11. Based on the number of hybridizing fragments and their size, the *SL8D* cluster contains at least four genes and probably many more if the possibility of overlapping

bands is considered. Some of the recently isolated *R* genes were also shown to be members of large, clustered gene families, but complete mapping data for them are lacking. The complex pattern of distribution of *I2C* is reminiscent of the case of the *L* and *M* rust resistance genes of flax (Ellis et al., 1995). *L* appears to be a single multiallelic gene, whereas homologous sequences map to a more complex *M* locus containing a gene cluster. Both loci may contain *R* genes specific to different races of the same pathogen or to different pathogens.

An *I2C* homolog on chromosome 9 (*SL8B*) maps near the *Tm-2a* and the *Frl* resistance loci, whereas the *I2C* cluster *SL8C* maps in the vicinity of the *Sm* resistance gene. Interestingly, no member of the *I2C* family maps to the *I* locus on the short arm of chromosome 11, which confers resistance to *F. o. lycopersici* race 1, or the *I3* locus in chromosome 7, which confers resistance to races 1, 2, and 3 of *F. o. lycopersici*, suggesting that different *F. o. lycopersici* race specificities of *R* genes arose from distinct gene families rather than from evolution of a single gene family. The lack of an *I2C* member at the *I3* locus is of particular interest because it indicates that the evolution of two distinct *F. o. lycopersici* race 2 resistance genes occurred simultaneously and also suggests the presence of more than one avirulence gene in *F. o. lycopersici.*

Structural Properties of *I2C* and Their Relationship to Known *R* Genes

Adjacent to the N-terminal part of the *I2C* gene family are several motifs common to the *NB-LRR* group of plant *R* genes. One clearly defined functional domain is the nucleotide binding motif, which contains a P-loop sequence (box I; Saraste et al., 1990). The consensus sequence GMG-GαGKTT, where α designates an aliphatic amino acid, is highly conserved among the P loops of all *NB-LRR R* genes reported thus far. Residues 623 to 645 of *I2C-1*, included in the LRR region, fit the consensus for a leucine zipper (Busch and Sassone-Corsi, 1990). However, sequences of this nature are abundant in the databanks, and the existence of this consensus does not necessarily imply a function.

The C-terminal part of the *I2C* genes is composed of LRRs, a motif common to several classes of plant *R* genes. The LRRs of four members of the *I2C* family are highly homologous but differ from each other mainly by insertions or deletions. This may indicate evolutionary processes and hint at mechanisms that generate new *R* gene specificities, such as duplications, unequal crossing over, or slippage during DNA replication. Analysis of the genomic organization of the disease-related LRR protein from tomatoes suggests that a duplication mechanism was involved in the evolution of LRRs (Tornero et al., 1996). LRRs have been described as being involved in many protein–protein interactions (Kobe and Deisenhofer, 1995a). Thus, the LRR region may be responsible for the specificity of an interaction, with either a component from the pathogen or downstream factors being involved in signal transduction. The crystal structure of the LRR-containing protein porcine RNase inhibitor, and its associated RNase, has been described recently (Kobe and Deisenhofer, 1995b). The RNase inhibitor contains a horseshoe-like structure in which individual, 28- or 29-residue-long repeats constitute β and α hairpin units that are aligned parallel to a common axis (Kobe and Deisenhofer, 1993). However, the LRR consensus and length of *I2C* and of the other *NB-LRR*–type *R* genes differ from that of the RNase inhibitor, which may imply that they contain a less organized or different structure than that found for the RNase inhibitor.

Table 1. Antisense Suppression of *F. o. lycopersici* Race 2 Resistance in T_1 Transformants of the Cultivar Motelle[a]

Transgene	No. of Independent Transformants	R:S 3:1[b]	R:S 1:1	R:S 1:3
I2C-1 antisense	3	1	1	1
I2C-3 antisense	18	7	7	4
I2C-1 sense	2	0	1	1

[a] Transgenes containing partial segments of *I2C* members were expressed in Motelle cultivars resistant to *F. o. lycopersici* race 1 and 2. The rates of resistance to susceptibility (R:S) for this cultivar are > 9:1.

[b] Rates of susceptibility to *F. o. lycopersici* race 2 were tested by inoculating 30 seedlings from each independent transformant. The results obtained for each independent transformant are divided into the nearest of three susceptibility classes, as indicated. The highest expected theoretical rate for T_1 transformants containing one transgene that are rendered completely susceptible is 1:3.

Table 2. Proportion of *F. o. lycopersici* Race 2–Resistant T_1 Progeny in Selected VF36 Lines Carrying the *I2C-1* Transgene

Line	No. of Experiments/ Total No. of Plants	%R[a]	Significance[b]
VF36[c]	3/93	10	
VF36-134H(7)	3/164	36	<0.001
VF36-134H(2)	2/95	36	<0.001
VF36-134H(8)	3/118	37	<0.001

[a] %R, the percentage of plants found to be totally free of trace infection symptoms after they had been dissected.

[b] Significance indicates the probability that %R of each transgenic group tested is the same as %R of VF36 based on the chi square test.

[c] The VF36 control is *F. o. lycopersici* race 1 resistant and *F. o. lycopersici* race 2 sensitive.

Involvement of *I2C* Genes in *F. o. lycopersici* Race 2 Resistance

Expression of partial gene fragments of two members of the *I2C* family in a sense or antisense orientation abrogated

immunity to *F. o. lycopersici* race 2. The fact that only race 2 immunity was affected is consistent with evidence that race 1 resistance (*I*) maps to the short arm of chromosome 11, thereby being distinct from *I2*, and that the *I2C* family has no mapping homologs in that region. The expression of antisense transgenes clearly implicates *I2C*-like genes as being involved in *F. o. lycopersici* race 2 resistance. The demonstration of specific sense and antisense suppression of plant *R* genes is important, and this approach should facilitate *R* gene characterization. Similar sense suppression was also observed in complementation of the *rps2* mutation (Mindrinos et al., 1994). The otherwise completely normal development of the antisense plants under standard growth conditions suggests that resistance is the main biological function of the *I2C* family. It will be interesting to see whether this conclusion can be generalized to other plant *R* genes.

The proportion of *F. o. lycopersici* race 2–resistant seedlings was significantly enhanced in the progeny of transgenic plants containing the *I2C-1* transgene from the *SL8D* locus. However, the gene did not confer the expected complete resistance that would be expected for a dominant-type gene. If one member of the *I2C* family from the *SL8D* locus exists that is capable of conferring a complete dominant-type resistance, it would mask the partial resistance conferred by *I2C-1*. However, it is possible that none of the family members individually is sufficient to confer the dominant-type *F. o. lycopersici* race 2 resistance, implying that resistance necessitates the concerted expression of several genes from the family either for dosage effect or to present a broader repertoire of interactions. Interestingly, the intact *I2* locus displays behavior closer to partial dominance rather than to dominance. Thus, a threefold increase in disease rate in heterozygote (I2i2) versus homozygote (I2I2) plants was observed (Sarfatti et al., 1989). This observation is consistent with a dose-dependent component in the nature of *I2* expression. Characterization of all of the *I2C* members from the *SL8D* locus and extensive complementation tests are necessary to ascertain the precise nature of this locus.

Mechanisms thought to be involved in resistance to vascular wilt diseases, for example, tylose formation, differ from those implicated in leaf diseases involving the HR. Strikingly, despite the apparent differences in these downstream resistance mechanisms, the *I2C* family belongs to the same superclass of *R* genes described for nonvascular leaf diseases. The structural commonalties between *R* genes involved in these distinct types of resistances raise the question of whether the choice of defense strategy is dependent on the tissue that modulates the response or on the specific *R* gene–avirulence factor interaction. Functional dissection of the different gene domains, and their tissue-specific expression, will shed light on these issues. The existence of many independent large multigene families raises the question of the gene population size necessary to maintain a plant's resistance profile. Examination of complex gene arrays, such as *I2C*, will address this question and contribute to the emerging elucidation of *R* gene function.

METHODS

Plant Material and Genetic and Physical Mapping

Four *Lycopersicon esculentum* segregating F_2 populations were used for genetic mapping. In each case, an initial cross was made between a parent resistant to *Fusarium oxysporum* f sp *lycopersici* race 2 and a susceptible parent. The resistant and susceptible parental pairs for the first three populations were, respectively, cultivars Motelle and Money Maker, cultivars Mogeor and Vendor, and cultivars Motelle and LA1113 (chromosome 11 marker stock; kindly provided by C.M. Rick, University of California, Davis). The fourth population was initiated from the commercial hybrid line Br5577 as F_1 progeny. The F_2 plants (1200) were screened individually for recombinations between *TG105* and *TG36*. F_3 seedlings from each of the recombinant plants were screened to fix the recombination to a homozygous state. In this process, an additional 400 F_3 plants from 53 different families were screened. Restriction fragment length polymorphism (RFLP) analysis and *F. o. lycopersici* inoculation were performed as previously described (Segal et al., 1992). *F. o. lycopersici* colonization was assayed 2 weeks after inoculations, as described by Grinstein et al. (1984).

Yeast DNA for pulsed-field gel electrophoresis analysis was digested with limiting amounts of the restriction enzymes MluI, XhoI, and SalI to obtain successive partial digestions. Additional digests included the rare cutters SgrAI and PmeI. The digests were fractionated on counter-clamped homogeneous electric field gels (Bio-Rad), blotted, and hybridized with probes.

Genomic and cDNA Libraries and Sequence Analysis

Yeast artificial chromosome YAC340-G3 (Cornell University collection), which contains the RFLP marker *TG105*A, was generated from the *F. o. lycopersici* race 2–resistant tomato line Rio Grande *PtoR* and cloned in the vector pYAC4 (Martin et al., 1992). Probes from YAC340-G3 that were used for the genetic and physical mapping are as follows. D2 is from a genomic λ library of the tomato line VFNT Cherry selected during chromosome walking from *TG105*. *SL8*, *SR8*, and *6-16* are subclones of λEMBL3 clones from a library of the yeast line containing YAC340-G3. TAO1-1 is a cDNA clone selected by YAC340-G3 from a cDNA library from roots of the *F. o. lycopersici* race 2–resistant tomato *L. esculentum* cv Mogeor (Ori et al., 1997). The cDNA libraries were all constructed in λgt10 from roots or leaves of resistant-type *L. esculentum*. Cosmid clones were isolated from a genomic library of the *F. o. lycopersici* race 2–resistant variety *L. e.* Mogeor and constructed in the cosmid TDNA004541 (Jones et al., 1992). Cosmid and cDNA clones were sequenced with an automated sequencer (Applied Biosystems, Foster City, CA). Sequence analysis and alignments were performed using the sequence analysis software package of the Genetics Computer Group (Madison, WI; Devereux et al., 1984) and the Multiple Alignment Construction and Analysis Workbench (or MACAW) program (Schuler et al., 1991; Lawrence et al., 1993).

Plasmid Constructs and Plant Transformations

Subclone 134H was created by cloning a HincII fragment from cosmid I2C-134, containing ~3 kb upstream and 800 bp down-

stream to the *I2C-1* open reading frame, into the HpaI site of the pGA492 vector (An, 1987). Cosmid I2C-134 was also transformed into plants directly.

For the antisense experiments, a HindIII fragment containing nucleotides 2540 to 3716 of the *I2C-1* gene, in both orientations, and the cDNA clone *I2C-3* were subcloned downstream of the cauliflower mosaic virus 35S promoter fused to the translation enhancer Ω (Gallie et al., 1987) and upstream of the nopaline synthase terminator. The resulting cassettes were subcloned into the XbaI site of the pGA492 vector to yield constructs 2-1-4 (*I2C-1*, sense orientation), 6-3-3 (*I2C-1*, antisense orientation), and 31-17 (*I2C-3*, antisense orientation). Constructs were transferred by electroporation into *Agrobacterium tumefaciens* EHA101. Agrobacterium-mediated tomato transformations were performed with the *L. esculentum* cvs Money Maker, Motelle, and VF36, according to McCormick (1991).

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