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Identification of Regions in Alleles of the Flax Rust Resistance Gene *L* That Determine Differences in Gene-for-Gene Specificity

Jeffrey G. Ellis,[a,1] Gregory J. Lawrence,[a] Joanne E. Luck,[b] and Peter N. Dodds[a]

[a] Commonwealth Scientific and Industrial Research Organization–Plant Industry, GPO Box 1600, Canberra, ACT, 2601, Australia

[b] Cooperative Research Center for Plant Science, GPO Box 1600, Canberra, ACT, 2601, Australia

Thirteen alleles (*L*, *L1* to *L11*, and *LH*) from the flax *L* locus, which encode Toll/interleukin-1 receptor homology–nucleotide binding site–leucine-rich repeat (TIR-NBS-LRR) rust resistance proteins, were sequenced and compared to provide insight into their evolution and into the determinants of gene-for-gene resistance specificity. The predicted L6 and L11 proteins differ solely in the LRR region, whereas L6 and L7 differ solely in the TIR region. Thus, specificity differences between alleles can be determined by both the LRR and TIR regions. Functional analysis in transgenic plants of recombinant alleles constructed in vitro provided further information: *L10–L2* and *L6–L2* recombinants, encoding the LRR of *L2*, conferred *L2* resistance specificity, and an *L2–L10* recombinant, encoding the LRR of *L10*, conferred a novel specificity. The sequence comparisons also indicate that the evolution of *L* alleles has probably involved reassortment of variation, resulting from accumulated point mutations, by intragenic recombination. In addition, large deletion events have occurred in the LRR-encoding regions of *L1* and *L8,* and duplication events have occurred in the LRR-encoding region of *L2*.

INTRODUCTION

Genetic analysis of plant–pathogen interactions frequently shows that for each host plant gene for resistance, there is a corresponding gene in the pathogen, an avirulence gene, that determines pathogenicity. This gene-for-gene interaction, which was first described for the flax–flax rust (*Linum usitatissimum–Melampsora lini*) interaction and subsequently found in many other plant pathogen interactions (reviewed in Crute and Pink, 1996), has led to a model in which plant resistance genes are postulated to encode receptors for the direct or indirect products of avirulence genes in the pathogen (Ellingboe, 1980). The basis of the specificity of recognition in such interactions is a major focus of research.

Since 1994, a rapidly increasing number of plant disease resistance genes have been cloned (reviewed in Bent, 1996), including closely related rust resistance genes from the *L* and *M* loci of flax (Lawrence et al., 1995; Anderson et al., 1997). The flax *L* and *M* genes, the tobacco virus resistance gene *N* (Whitham et al., 1994), and the Arabidopsis downy mildew resistance gene *RPP5* (Parker et al., 1997) encode proteins belonging to a subgroup of resistance proteins called TIR-NBS-LRR proteins (Baker et al., 1997). These proteins consist of three domains: an N-terminal domain related in sequence (Whitham et al., 1994) and in predicted secondary structure (Parker et al., 1997) to the cytoplasmic domain of Toll proteins and the interleukin-1 receptor protein (TIR), a central nucleotide binding site (NBS) domain, and a C-terminal leucine-rich repeat (LRR) domain.

Because LRR proteins are frequently involved in protein–protein interactions (Kobe and Deisenhofer, 1994), such as specific interactions between mammalian hormone receptors and their glycoprotein ligands (Braun et al., 1991), it has been proposed that the LRR region of resistance proteins may be involved in the specificity of gene-for-gene interactions (Staskawicz et al., 1995). In the Cf-4 and Cf-9 resistance proteins of tomato, which belong to a different class of resistance proteins and consist of only a membrane-bound extracellular LRR domain, the specificity difference between the two proteins is determined by differences within the 454 amino acids of the N-terminal half of the molecule (Thomas et al., 1997). The majority of these differences occur in the conserved β strand–β turn structural motif of LRR units. Analysis of nucleotide substitution rates suggests that this region of the LRR units in 11 *Cf-9*/*Cf-4* paralogs is subject to diversifying selection (Parniske et al., 1997), which is consistent with this region being involved in specificity determination.

[1] To whom correspondence should be addressed. E-mail jeff.ellis@pi.csiro.au; fax 61-2-6246-5000.

In flax, the series of rust resistance alleles at the *L* locus provides ideal genetic material for the analysis of the molecular basis of gene-for-gene specificity. Thirteen alleles of the *L* gene have been described, with each conferring a different rust resistance specificity. Flax plants expressing these alleles can be distinguished by their reaction (resistance or susceptibility) to a range of flax rust strains with and without the corresponding avirulence genes (reviewed in Islam and Mayo, 1990). A fourteenth allele, *LH*, occurs in the cultivar Hoshangabad, which is susceptible to all strains of flax rust (Lawrence et al., 1995).

In this study, the DNA sequences of 13 *L* alleles are described, and evidence is presented that specificity is determined by their coding regions. Comparisons of the predicted amino acid sequences of allelic products and in vitro intragenic exchanges involving the *L2*, *L6*, and *L10* alleles are used to identify the TIR and LRR regions as determinants of the specificity differences between several alleles. In addition, DNA sequence analysis shows that some alleles have undergone large duplication or deletion events in the LRR-encoding region and that the alleles have a mosaic nature, probably due to reassortment of variation present in ancestral alleles by recombination.

RESULTS

Cloning Alleles of the Flax Rust Resistance *L* Gene

Twelve alleles (*L*, *L1* to *L5*, *L7* to *L11*, and *LH*) of the flax rust resistance gene *L* were cloned. Genomic libraries made from flax lines homozygous for the *L2*, *L10*, or *LH* alleles were screened with the probe *Lu-1* (Ellis et al., 1995), derived from the promoter region of the previously cloned *L6* allele (Lawrence et al., 1995). The remaining nine alleles were amplified by long-range polymerase chain reaction (PCR) by using DNA isolated from flax lines homozygous for each of these alleles and cloned in plasmid vectors. PCR primers were based on DNA sequence from upstream and downstream of the coding region of the *L6* allele.

Transformation Confirms That Rust Resistance Specificity Is Determined by Alleles of the *L* Gene

Before proceeding with the complete sequence analysis of the alleles, we conducted experiments to determine whether these sequences indeed encode gene-for-gene resistance specificity. Genomic clones of the *L2*, *L6*, and *L10* alleles and a cDNA clone of *L6* expressed using the cauliflower mosaic virus 35S promoter were introduced into flax by Agrobacterium-mediated transformation, and the resulting transgenic plants were inoculated with flax rust strains to determine whether the cloned alleles control specific rust resistance. The *L2* clone was used to transform the flax line Forge, which does not express the *L2* specificity. Two transgenic plants were resistant to rust strain Sp-y, which is avirulent to *L2* but virulent to Forge. A similar experiment was performed with the cloned *L6* gene. One transgenic plant containing *L6* was recovered after transformation of Hoshangabad, a flax line that is susceptible to all strains of flax rust. This plant was resistant to rust strain CH5F2-84, which is avirulent to *L6.* Transformation of the flax line Ward with *L10* gave rise to four transgenic plants that were resistant to the rust strain BS-1, which is virulent to Ward but avirulent to plants carrying the *L10* gene. The resistance phenotypes of all the transgenic plants were completely unambiguous; small hypersensitive flecks appeared on infected leaves, and no uredospore formation occurred. In contrast, inoculation of susceptible plants gave rise to infections producing pustules bearing copious orange uredospores.

Furthermore, plants expressing the *L2*, *L6*, and *L10* transgenes, like those transformed with the closely related *M* gene (Anderson et al., 1997), were susceptible to rust strains not carrying the corresponding avirulence genes, thus demonstrating that each transgene confers gene-for-gene specificity.

In addition to genomic clones, a 35S–*L6* cDNA construct was used to transform the flax line Trac1, which is homozygous for an allele of *L6* inactivated by the *Activator* transposon and thus lacks *L6* rust resistance. Three transgenic plants were resistant to rust strain CH5F2-84, which is virulent to Trac1 but avirulent to *L6* plants. The resistance phenotype of 35S–*L6* cDNA plants was indistinguishable from plants containing the unmodified *L6* gene. A line homozygous for the 35S–*L6* cDNA transgene was derived from one of the transgenic lines. This line was resistant to rust strain CH5F2-84, which carries *A-L6*, the avirulence gene corresponding to *L6*, and was susceptible to strain Sp-y, which does not carry *A-L6.* The transgenic plants also were examined by the gel blot analysis outlined in Lawrence et al. (1995) to confirm that the resistance expressed by these plants was not due to excision of *Activator* from the mutant *L6* gene in the Trac1 line used for transformation. Because the 35S–*L6* cDNA contains only a small region of untranslated sequence, the experiment points to the coding region of *L6* as being the determinant of *L6* resistance specificity.

For each cloned allele and for the 35S–*L6* chimeric gene, one primary transgenic plant, identified by gel blot analysis as having a single transgene insert, was selected for genetic analysis. In each case, the selfed progeny (from 12 to 24 per parent) segregated resistant-to-susceptible progeny consistent with a 3:1 ratio, and complete cosegregation of specific rust resistance and the transgene, detected by DNA gel blot analysis, was obtained.

Sequence Variation of *L* Alleles

The DNA sequences of 12 alleles and that of the previously sequenced *L6* allele were compared to assess the degree of

sequence variation between alleles and to identify potential specificity-determining regions. The alleles were sequenced from the conserved SacI site 162 bp upstream of the ATG translation initiation codon to a conserved BglII site ~35 bp downstream of the stop codon. The DNA sequences were all >90% identical to *L6*, but each contained several differences, with the exception of *L3*, *L4*, and *L10.* In addition, the sequences of the three introns were either identical in each allele or differed from *L6* by at most two residues.

Unexpectedly, the DNA sequences obtained for *L3* and *L10* were identical. *L4* differed from *L3* and *L10* by a single nucleotide that caused an amino acid substitution. Plants carrying these three alleles had originally been distinguished by their reactions (more or less resistance) to various strains of rust (Flor, 1947, 1955), but no rust strains with reciprocal reactions (i.e., avirulent on one and virulent on the others and vice versa), which would unambiguously indicate different specificities, have been described. The observation that *L3* and *L10* encode identical products raises the possibility that background genetic variation in *L3* and *L10* plant lines, including variation that may occur in the unsequenced promoter regions, affects reaction to rust and thus permits these lines to be distinguished. For *L4*, the single amino acid difference may be sufficient for the reported specificity, or again, background genetic effects may distinguish rust reactions on *L4* plants. If the *L* alleles in these three lines do encode the same specificity, the gene-for-gene theory predicts that *L3*, *L4*, and *L10* should interact with the same avirulence gene in rust. Evidence consistent with this is provided by the results of Flor, who found that the avirulence genes in flax rust (*AL-3*, *AL-4*, and *AL-10*) that correspond to *L3*, *L4*, and *L10* map at the same locus, whereas most other avirulence genes in flax rust are not closely linked (reviewed in Lawrence, 1988). Therefore, we conclude that *L3*, *L4*, and *L10* may determine the same specificity.

Three other alleles, *L5*, *L6*, and *L7*, also interact with avirulence genes that map to a single locus in flax rust (*AL-5*, *AL-6*, and *AL-7*; reviewed in Lawrence, 1988). In this case, however, the sequence of *L5* is distinct from *L6* and *L7* throughout the sequenced region. However, although having a distinct exon 1 sequence, *L6* and *L7* are identical in the remaining part of the gene. Nevertheless, strains of flax rust that clearly differentiate these alleles have been reported (Flor, 1955, 1956; Timmis et al., 1990), which indicates that *L5*, *L6*, and *L7* encode distinct gene-for-gene specificities.

The *LH* allele occurs in the flax line Hoshangabad, which is susceptible to all tested flax rust isolates from cultivated flax and from its Australian wild relative *L. marginale.* Transformation of Hoshangabad with *L6* nevertheless gave rise to a resistant transgenic plant (see above), which indicates that susceptibility of this line is due to the *LH* allele and not mutation in signal pathway genes. The sequence analysis of *LH* does not identify any apparent feature that accounts for the inactivity of the allele. It contains no insertion sequence or "footprint" indicative of transposon mutagenesis, no deletion or point mutation that would give rise to premature stop codons, and no major sequence changes in recognized functional motifs such as the P loop and kinase-2a motif of the NBS region. Furthermore, in vivo recombinants that contain the 5′ promoter region and exon 1 of *LH* and the downstream region of *L6* provide rust resistance (J.E. Luck, unpublished results). This indicates that these upstream regions of the allele, including its promoter, are functional, and suggests that sequence differences between *LH* and other alleles downstream of exon 1 either prevent gene function or prevent specific recognition of any rust avirulence gene assayed. It is also noteworthy that the *L9* allele in the cultivar Bison, which is susceptible to all American isolates of flax rust, confers resistance to some Australian rust isolates. Therefore, in the absence of an appropriate rust strain, a functional resistance allele may appear nonfunctional.

Structural Variation among *L* Alleles

The structural arrangement of all but three alleles is identical to that of *L6*: they have approximately the same length and have two directly repeated sequences of ~450 bp in the LRR-encoding exon 4. A similar direct repeat occurs in the related flax rust resistance gene *M* from the unlinked *M* complex (Anderson et al., 1997). The first and second repeat units in the *M* gene and in the *L* alleles can be distinguished by the presence of unique sequence motifs of 18 bp (motif 1) and 24 bp (motif 2) (Anderson et al., 1997) near the beginning of the first repeat and near the end of the second, respectively (indicated as vertical bars in Figure 1). This fact is important in interpreting the structure of the three alleles *L1*, *L2*, and *L8*, which have a very different structure from *L6* (Figure 1). *L1* and *L8* have undergone internal deletions in the region encoding the LRRs. In the case of *L1*, there are two internal deletions in exon 4. The first is of 525 bp and occurs upstream of the two large repeated units. The second, of 429 bp, deletes the end of the first direct repeat and the beginning of the second, with the result that a single repeat unit containing both motifs 1 and 2 mentioned above is present in *L1.* A similar event, which is the result of intragenic sequence exchange, was observed in three spontaneous mutants of *M* and resulted in the loss of *M* specificity (Anderson et al., 1997). In *L8*, a single deletion of ~1434 bp begins in exon 3, includes intron 3, and extends to near the end of the first direct repeat. Although this allele contains a single repeat unit, it is structurally identical to the last repeat in an *L6*-like allele in that it contains only the 24-bp motif. The internal deletions in *L1* and *L8* do not alter the reading frames, and the alleles encode internally deleted proteins (see Figure 2).

In the *L2* allele, sequence duplication has occurred; there are four copies of the direct repeat unit (Figure 1). The first and fourth repeats in *L2* are similar to the first and second *L6* repeats, respectively, in that they contain the appropriate unique motif 1 or 2. However, the two internal repeat units lack both motifs 1 and 2 and differ from each other at only

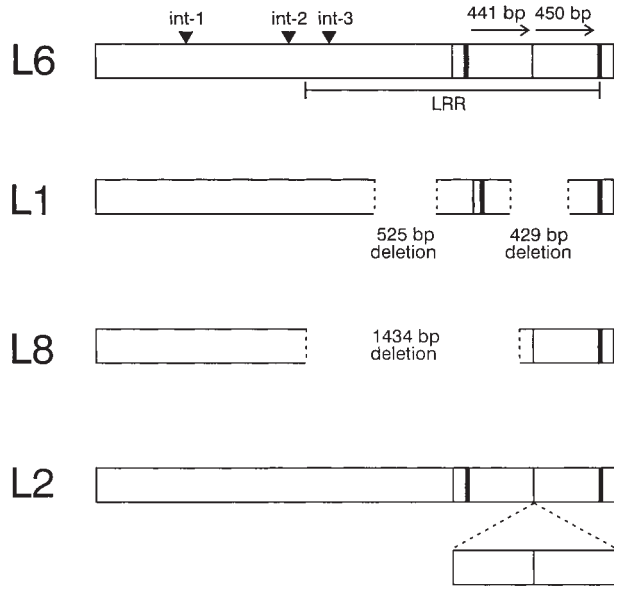

Figure 1. Major Structural Changes in *L1*, *L2*, and *L8.*

The coding regions of the *L1*, *L2*, and *L8* alleles are compared with that of *L6.* The regions deleted in *L1* and *L8*, indicated by gaps in the bars, were detected by alignment of both sequences to *L6* by using the BESTFIT program (Devereux et al., 1984). The locations of the conserved introns (int) are indicated by arrowheads, the LRR region is marked under *L6*, and the positions of the direct repeats are indicated by arrows above the alleles. The unique 18- and 24-bp sequences in the first and last direct repeat units are shown by black bars. The two additional central repeats in *L2*, indicated beneath the *L2* bar, lack these sequences.

five of 426 nucleotides. The expansion of the repeat region could have occurred by two steps of unequal crossing-over and subsequent point mutation to introduce the minor sequence differences. Unequal crossing-over between the first repeat of one gene and the second repeat of another would give rise to a product with one repeat and a second product with three. Subsequent unequal crossing-over between two genes with three repeats (or one gene with three and a second with two repeats) could have given rise to the four-repeat structure of *L2.*

Amino Acid Sequence Comparisons of *L* Allele Products

The amino acid sequences of 11 of the allelic products were aligned for comparison (Figure 2). For this comparison, the two central members of the four repeat units in the LRR region of *L2* have been removed from the sequence to facilitate the alignment. There are 242 positions in the 1294 to 1304 amino acids that are variable; at 177 of these positions, two or more alleles differ from the others. At the majority of variable positions, only two alternative residues (or deletion of a residue) occur, and at only 58 positions are there more than two alternative residues. Overall, the products of the *L* alleles are very similar. Six percent of the positions in the product of exon 1 are variable, whereas 13, 34, and 23% of the positions in the products of exons 2, 3, and 4, respectively, are variable. Thus, the greatest variation occurs in the LRR region encoded by exons 3 and 4.

Six short "hypervariable" domains have been distinguished (A to F in Figure 2). These domains consist of six to 11 amino acids in which almost all positions are polymorphic and which contain several positions with more than two alternative residues. Two variable regions occur in the exon 3 product. Region A, which occurs 10 residues upstream of the start of the LRR (Lawrence et al., 1995; Jones and Jones, 1997), includes short deletions and/or insertions in several allele products. L5 and L10 contain an identical region A sequence of 11 residues. The *L* allele product contains a similar sequence of only nine amino acids (three differences from L5 and L10), and in the other allelic products, region A contains only four residues. This site is also highly variable in the product of the *M* rust resistance gene of flax, which contains a 10–amino acid sequence different from all *L* allele proteins (Anderson et al., 1997). A second protein, FC4, encoded by a member of the *M* gene cluster, also has a unique sequence in this region. Region B occurs five residues downstream of the beginning of the LRR and also varies among the products of the *M* locus members *M* and *FC4*, as do regions C and D near the beginning of the exon 4–encoded LRR and regions E and F that occur near the C terminus of the proteins.

Examination of the amino acid alignment (Figure 2) indicates a nonrandom distribution of variation throughout the LRR. Much of this variation occurs in the potential solvent-exposed β strand–β turn structural motifs (underlined in Figure 2) postulated for the L6 sequence by Jones and Jones (1997). However, some of the variation, for example, in hypervariable regions C and D, occurs outside of the proposed β strand–β turn structural motifs, and in addition, some of the β strand–β turn regions are conserved among all L proteins. Superimposition of the proposed β strand–β turn motifs on an amino acid alignment of the L6 and M flax rust resistance proteins presented in Anderson et al. (1997) provides similar results for the distribution of variation between the two proteins. The issue raised by these sequence comparisons is whether the variation has been subject to selection. This issue, first addressed for resistance genes by Parniske et al. (1997) for the *Cf* genes in tomato, has been considered recently for NBS–LRR resistance genes. Meyers et al. (1998) performed a pairwise analysis of the ratio of synonymous to nonsynonymous substitutions in the nucleotide sequences of the closely related *L6* and *M* flax rust resistance genes. Their analysis indicated that diversifying selection has acted on the regions encoding β strand–β turn

motifs but not on the regions of the LRR coding sequences between the β strand–β turn motifs.

Evolution of *L* Alleles by Recombination

Examination of the polymorphic positions in the amino acid sequences (Figure 2) and the DNA sequences (Figure 3) shows that certain groups of alleles are closely related in some regions and then diverge. For example, in the TIR domain, L7 and L10 proteins are closely related, with only a single amino acid difference. In the same region, L6 and L7 differ by 11 amino acid residues. However, the NBS and LRR domains of L6 and L7 are identical and different from L10. This suggests that the *L6* and *L7* alleles may have diverged from each other by a single recombination event near the end of exon 1. A similar event may have occurred for *L6* and *L11.* The amino acid sequences encoded by these two alleles are identical in the TIR and NBS domains, differ by a single amino acid in the product of exon 3, and then diverge, particularly near the end of the first direct repeat unit in the LRR. At this point, the sequence of the L11 protein most closely resembles L5. Further evidence for such a recombination event in the evolution of *L6* and *L11* is provided by the presence of two *L6*/*L11*–specific polymorphisms (the absence of an EcoRV site and a 45-bp deletion) in the 5′ noncoding region (data not shown).

Other alleles have more complex relationships. Figure 3 shows an alignment of informative polymorphic sites (IPSs), as used by Parniske et al. (1997) for tomato *Cf* gene analysis. These sites have two or more alternative nucleotides, with each occurring in at least two sequences, and therefore can be used to infer phylogenetic relationships. This alignment indicates that multiple sequence exchange events may have occurred during the evolution of the alleles so that any allele contains sequential segments related to different alleles. For example, beginning in the NBS region, *L9* and *L1* share IPSs (and differ from other alleles) over a region of 793 bp; farther downstream near the beginning of the LRR region, *L9* and *LH* share IPSs over 90 bp; farther downstream again in the LRR region, *L9* and *L2* share IPSs over 236 bp; and then, *L9* shares IPSs with *LH* over a region of 1023 bp. These IPSs include synonymous substitutions that counter the alternative hypothesis that these shared regions of similarity result from convergent evolution.

Analysis of in Vitro Sequence Exchanges between *L* Alleles

In an effort to identify DNA regions that determine the specificity differences between alleles, recombinant genes involving *L2*, *L6*, and *L10* alleles were constructed in vitro (Figure 4). Eight different gene constructs were prepared. These chimeras and the positions of the restriction sites used for the exchanges are illustrated in Figure 4. The locations of exchanges with respect to structural motifs in the L proteins also can be seen in the amino acid sequence comparisons in Figure 2. These constructs were introduced into the flax line Ward, and the transgenic plants were tested for resistance to rust strains that distinguish the *L2*, *L6*, and *L10* specificities. All rust strains used in these experiments were virulent to Ward.

Sequence Exchanges at the Conserved SphI Restriction Site near the 3′ End of Exon 2

Four recombinant genes were made by using the conserved SphI site that occurs in exon 2, which is 70 bp upstream of the DNA sequence encoding the GLPL amino acid motif conserved in NBS-LRR resistance proteins (Figure 2; Staskawicz et al., 1995). Approximately 413 amino acid residues are encoded by the sequence upstream of this site, and at least 880 (more for *L2* with its four repeats), including the entire LRR and six hypervariable sites, are encoded downstream of the site.

Two independent transgenic plants containing *L6–L2Sph* were inoculated with rust strain CH5F2-133, which is avirulent to *L2* and virulent to *L6.* One plant was resistant. Cuttings from this plant were subsequently tested with rust strains CH5F2-87 and CH5F2-134, which are avirulent to plants carrying the *L6* gene and virulent to plants carrying *L2.* The cuttings were susceptible to both rust strains, which indicated that the chimeric gene did not express *L6* specificity. Therefore, the resistance specificity expressed by the chimeric gene in this plant is consistent with the *L2* specificity. The resistant plant was selfed, and among 18 progeny plants, 16 were resistant and two were susceptible to rust strain BS-1, which is avirulent on *L2.* The transgene, detected by DNA gel blot analysis, cosegregated with the rust resistance phenotype. Similar experiments were performed with *L10–L2Sph.* Among eight independent transgenic plants, four were resistant to rust strain CH5F2-133 that is avirulent to *L2* and virulent to *L10.* This result is consistent with the *L2* specificity being expressed by *L10–L2Sph.* Exact cosegregation of the rust resistance phenotype and the transgene was observed among the progeny of one of the resistant transgenic plants.

Among five independent transgenic plants containing *L2–L10Sph*, four plants were resistant to rust strain BS-1, which is avirulent to *L2* and *L10.* This result is consistent with the chimeric gene expressing either *L2* or *L10* specificity. One of the transgenic plants was subsequently tested with rust strain CH5F2-133, which is virulent to *L10* and avirulent to *L2.* The plant was susceptible and therefore did not express *L2* resistance. No suitable rust strain avirulent to *L10* and virulent to *L2* was available. Nevertheless, the results are consistent with *L2–L10Sph* encoding *L10* resistance specificity. However, plants containing *L2–L10Sph* were later found to be susceptible to several other rust strains

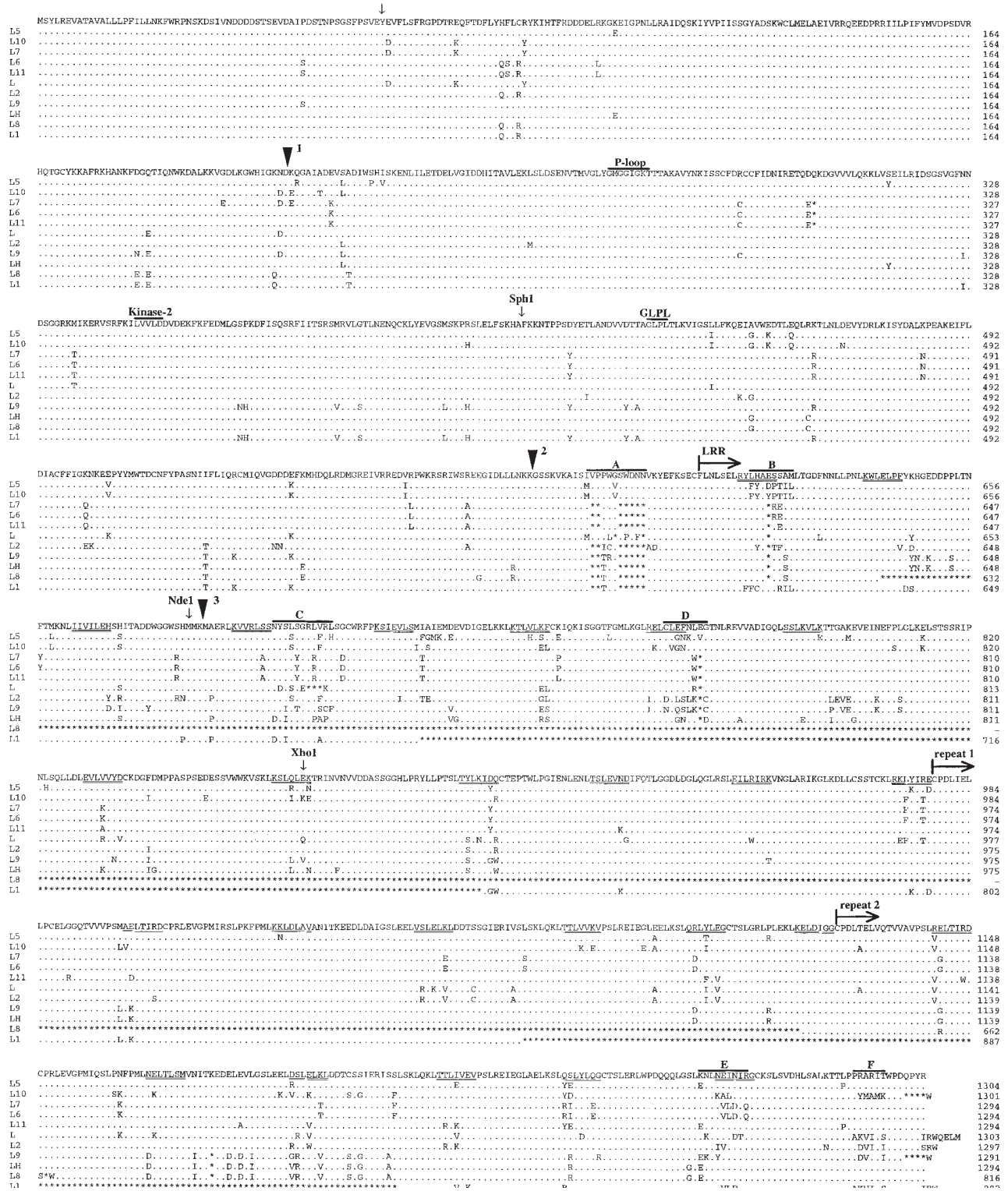

Figure 2. Amino Acid Sequence Alignments of Products of the *L*, *L1*, *L2*, *L5* to *L11*, and *LH* Alleles.

The amino acid sequences of 11 L polypeptides are shown compared with the consensus of these sequences (upper line), showing the most common residue at each position. The sequences of *L3* and *L4* have been omitted because *L3* is identical to *L10*, and *L4* differs from *L10* by a

that recognize the standard *L10* allele, which suggests that the chimeric gene may express a different specificity. These data are discussed further below. Again, progeny analysis of one of the resistant transgenics revealed exact cosegregation of the resistance phenotype and the transgene.

Thirteen independent transgenic plants containing *L6–L10Sph* were identified by gel blot analysis, and all were susceptible to rust strain BS-1, which is avirulent to *L6* and *L10.* This result was unexpected because the three previously described chimeric genes made at the SphI site were functional. Sequence analysis of the coding region of the chimeric gene indicated that no changes had been introduced during cloning steps. Furthermore, reverse transcriptase–PCR analysis of RNA from four of the transgenic plants detected transcription of the chimeric transgene in two plants (data not shown). Mutations in the coding region or lack of transcription of the chimeric gene are therefore not the cause of the lack of rust resistance in the transgenic plants.

Sequence Exchanges within the LRR Region Involving the NdeI Site in Exon 3 and the XhoI Site in Exon 4

The chimeric gene construct *L2–L10Nde* was made using an NdeI site in exon 3 that occurs 900 bp downstream of the SphI site used in earlier domain swaps and 10 bp upstream of the 5′ end of intron 3. This exchange occurs in the beginning of the LRR-encoding region and divides the variable regions A and B from the remaining hypervariable regions (Figure 2). Sixteen independent transgenic plants were tested for resistance to rust strain BS-1. None of the plants was resistant, indicating that they expressed neither *L2* nor *L10* resistance specificity.

Three additional chimeric genes were constructed involving *L2* and *L6* in which a central section of one gene was replaced with the corresponding section of the other gene. An XhoI restriction site that occurs in the LRR coding region of exon 4 of both *L2* and *L6* was utilized, in conjunction with one other restriction site (SacI, SphI, or BglII; see Figure 4). Seven independent transgenic plants containing *L6–L2–L6Sac/XhoI*, 11 containing *L2–L6–L2Xho/Bgl* (these two constructs encode identical proteins), and seven containing *L6–L2–L6Sph/Xho* were tested with the rust strain BS-1, which is avirulent to *L2* and *L6.* None of the transgenic plants was rust resistant, indicating that they expressed neither *L2* nor *L6* resistance specificity.

A Chimeric Gene with Altered Resistance Specificity

The *L2–L10Sph* chimeric gene, which as reported above does not express *L2* specificity, was tested with five rust strains with different genetic backgrounds but all avirulent to *L10.* These rust strains were used to inoculate T_1 progeny plants carrying the *L2–L10Sph* chimeric gene and three different lines carrying the standard *L10* allele (Bolley Golden selection, the *L10* gene from Bolley Golden selection transferred by six backcrosses into the cultivar Bison, and a line transgenic for the cloned *L10* gene introduced into cultivar Ward). In this experiment, all five rust strains were avirulent to the standard *L10* lines and to the transgenic *L10* line, confirming *L10* specificity. However, although BS-1 was avirulent, the other four strains were all virulent to *L2–L10Sph* transgenic plants. This result indicates that *L2–L10Sph* expresses a resistance specificity different from *L10.* Furthermore, because the four supplementary rust strains collectively recognize all previously described *L* specificities, the specificity expressed by *L2–L10Sph* is a novel *L* gene specificity.

DISCUSSION

Complementation Analysis with Cloned Rust Resistance Alleles

In a previous report (Lawrence et al., 1995), the cloning of the *L6* allele at the *L* rust resistance locus of flax by transposon tagging was described. Here, we show that transgenic flax plants containing the genomic or cDNA clone of *L6* confer *L6*-specific rust resistance. In addition, two *L* alleles from lines of the *L2* and *L10* genotype were cloned and also confer rust resistance of the appropriate specificity in transgenic

Figure 2. (continued).

single amino acid (D replaces N at residue 470). The numbers at the end of each line are the positions of the last residue in that line. Residues identical to the consensus are shown as dots, and deleted residues are indicated with asterisks. The P loop and kinase-2 motif of the NBS and the conserved GLPL motif of unknown function (Staskawicz et al., 1995) are labeled, and the start of the LRR region and of the two direct repeats are indicated by arrows (the two central repeats of the four repeats in *L2* are not included in the comparison). The positions of the three introns in the nucleotide sequence are shown as numbered arrowheads, and six hypervariable regions A, B, C, D, E, and F are overlined. The positions of the SphI, NdeI, and XhoI sites in the DNA sequences used for making sequence exchanges are shown. The inverted arrow at residue 61 indicates the end of the 60–amino acid extension in L proteins that is absent in the tobacco N and Arabidopsis RPP5 resistance proteins and also marks the beginning of the TIR. The predicted β strand–β turn motifs in the LRR are underlined.

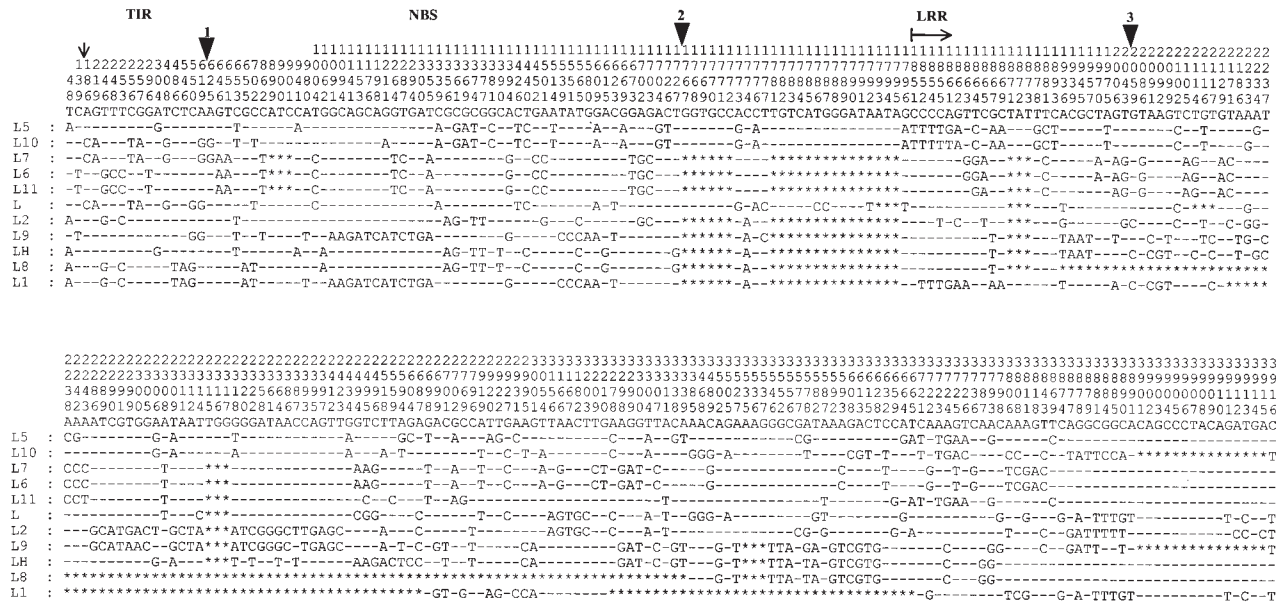

Figure 3. Alignment of IPSs in the DNA Sequences of *L* Alleles Indicates Evolution of Alleles by Segmental Exchange.

Only the IPSs of the nucleotide sequences of the coding regions of *L* alleles are shown. The nucleotide positions of each site are indicated above the consensus line. Nucleotides identical to the consensus line are indicated by dashes, and deleted nucleotides are denoted by asterisks. Vertical arrowheads show the intron positions, and the start of the LRR is indicated. The regions encoding the TIR and NBS domains are also labeled, with the start of the TIR indicated by a vertical arrow.

plants. These results confirm the identity of the cloned sequences as alleles of the *L* gene. As a consequence, 10 additional alleles of the *L* gene were cloned and sequenced to provide information on the evolution of alleles and the basis of their gene-for-gene specificity.

Evolution of *L* Alleles

The sequence analysis of the 11 alleles under comparison has indicated that eight are >90% identical. The differences have most likely arisen by point mutations over time that have resulted in changes in 244 of the ~1300 amino acids that constitute the "standard" gene product. These changes occur throughout the full length of the gene product with the greatest variation in the LRR region.

Major structural changes (duplications and deletions) were found to have occurred in the LRR regions of three alleles, *L1*, *L2*, and *L8.* Some of these events may have occurred as a result of unequal crossing-over between direct repeats in the LRR coding region. Similar changes have been observed in spontaneous mutants of the *M* rust resistance gene of flax (Anderson et al., 1997) and in a mutant of the *RPP5* downy mildew resistance gene of Arabidopsis (Parker et al., 1997). The former resulted in loss of rust resistance, whereas the latter did not alter resistance to downy mildew. These results indicate that repeated elements in resistance genes can mediate their evolution.

Comparison of the DNA sequences of the *L* alleles reveals a patchwork or mosaic of sequence similarities (see Figure 3). This observation suggests that extensive reassortment of variable regions within the alleles has occurred by multiple intragenic sequence exchange events. Comparisons of the most closely related *L* allele pairs, *L6* to *L7* and *L6* to *L11*, have identified likely single exchanges in exon 1 and exon 4, respectively. Gene conversion or sequential crossing-over events in successive generations could account for the mosaic nature of the *L* alleles. Indeed, Islam and Shepherd (1991) observed intragenic recombination at a frequency of approximately one per 1000 gametes from some *L* allele heterozygotes: some of the recombinant alleles expressed a resistance specificity different from the parental alleles, and in four cases we localized the cross-over points within the coding regions of the parental alleles (J.G. Ellis and G.J. Lawrence, unpublished data).

The original allelic variation that forms the substrate for recombination could be supplied by point mutations or transposon activity, and any specificity change that provides novel resistance would be selected by pathogen pressure. Thus, balancing selection would be expected to promote and maintain diversity at the *L* locus. Parniske et al. (1997) observed a similar patchwork of sequence relationships in

resistance genes at the *Cf-4*/*Cf-9* complex locus of tomato and attributed this to intergenic exchanges between related members of the gene cluster. The ability to copy this evolutionary process by genetic engineering to provide novel specificities for control of plant diseases in agriculture is a significant challenge.

Which Region or Regions Determine Specificity?

Several observations point to the LRR region as a major determinant of specificity differences between *L* alleles. First, most of the sequence variation between alleles occurs in this region. Of the six hypervariable regions identified (Figure 2), four occur within the LRR region, whereas the other two (A and F) are immediately adjacent to the LRR region. Much of the variation occurs within, or immediately adjacent to, the postulated solvent-exposed β strand–β turn structural motifs (Jones and Jones, 1997; see Figure 2). These were first characterized in the porcine ribonuclease inhibitor protein as the substrate binding domain (Kobe and Deisenhofer, 1995). Furthermore, similar to the observations of Parniske et al. (1997) for the *Cf-4*/*Cf-9* genes of tomato, an evolutionary analysis of the ratio of nonsynonymous to synonymous nucleotide substitutions in *L6* and the closely related *M* flax rust resistance gene recently published by Meyers et al. (1998) demonstrated that the postulated β strand–β turn motifs of the LRR region of these two genes have undergone diversifying selection. A similar analysis involving a pairwise analysis of the *L* alleles, to be published elsewhere, also indicates diversifying selection acting on the sequences encoding the LRR region of the *L* alleles (P.N. Dodds, unpublished data).

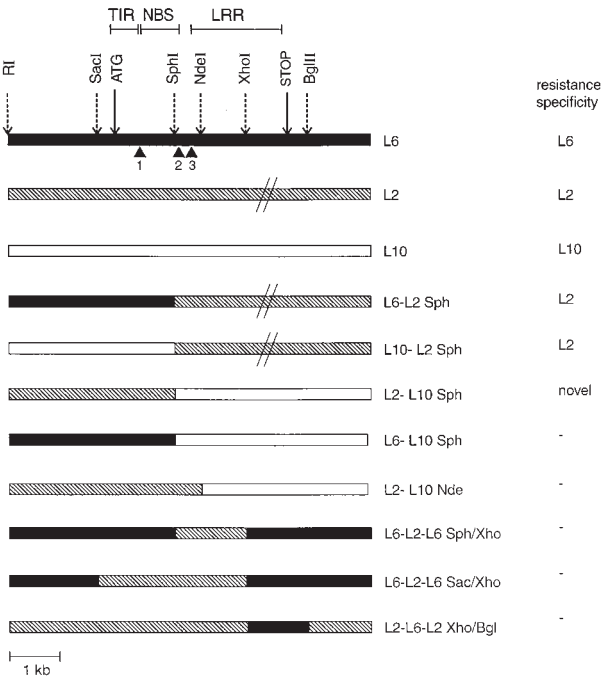

Figure 4. Rust Resistance Specificity of *L* Gene Constructs in Transgenic Flax.

Schematic diagram of *L6* (black), *L2* (striped), *L10* (white), and recombinant gene constructs used to transform flax. The restriction site suffixes of the chimeric genes indicate the positions at which the exchanges were made. The positions of restriction sites, beginning at the 5′ EcoRI site (RI), for cloning and in vitro intragenic exchanges are shown above the maps. The XhoI site is polymorphic and is absent from *L10*. The positions of the ATG and stop codons and the TIR-, NBS-, and LRR-encoding sequences are also shown. The position of the two extra repeat units in the *L2* sequence is indicated by double slashes. The positions of the three introns are shown by the numbered arrowheads. The specificity expressed by each allele in transgenic flax is indicated at right. Those that gave no resistance are indicated by a (−), and the modified resistance conferred by the *L2–L10Sph* alllele is indicated as "novel."

The second indication for the role of the LRR region comes from a comparison of the *L6* and *L11* alleles, which are identical over the first 620 amino acids and differ only in the LRR region. Thus, these differences must account for the difference between *L6* and *L11* specificity. However, the most direct evidence comes from in vitro sequence exchange experiments in which the *L2* LRR region was combined with the *L6* or *L10* N-terminal region, including the TIR and most of the NBS regions (Figure 4). Both chimeric alleles expressed *L2* rust resistance specificity but not *L6* or *L10* specificity. This demonstrates that the sequence differences between *L2* and *L6* and *L2* and *L10* downstream of the exchange point and including the LRR region are important for *L2* specificity.

In addition to sequence variation in the LRR, variation in its length may also contribute to specificity differences. For example, a deleted LRR region, including the first 32 amino acid residues of the LRR together with the last 150–amino acid direct repeat unit, is sufficient for *L8* specificity. The L8 protein retains the two hypervariable regions A and B just upstream and at the beginning of the LRR, respectively, and regions E and F at the C terminus of the protein. Conversely, four 150–amino acid repeat units occur in the LRR region of the L2 protein, whereas most L proteins contain only two of these repeats. The expansion or reduction of the distance between the variable regions may contribute to the evolution of new specificities in the host in a similar way that variation in the number of repeat units in the *avrBs3* class of bacterial avirulence genes changes avirulence specificity (Herbers et al., 1992).

Two observations indicate that the region at the N-terminal end of the product also plays a role in specificity determination. First, the products of *L6* and *L7* are identical, except for 11 amino acid differences that are all located within the first 208 amino acids of the N-terminal end (the TIR region). This has been confirmed by precise sequence exchanges involving just the TIR region (J.E. Luck, unpublished data). The second observation concerns the chimeric

allele *L2–L10Sph*, in which the region at the 5′ end of *L10* was replaced by the equivalent region of *L2*: the resulting gene expressed a new specificity. A chimeric gene containing the *L2* promoter and *L10* coding region has *L10* specificity (J.G. Ellis, unpublished data), indicating that the specificity change in *L2–L10Sph* is also due to changes in the 5′ coding region.

It is important to note, as discussed by Verica et al. (1998) and Matton et al. (1998) in relation to self-incompatibility genes, that sequence comparisons and in vitro exchanges between pairs of alleles can only address the role of the sequence differences in specificity detemination. The contribution of shared sequences to their respective specificities cannot be assessed, and specificity differences between other alleles may be determined by different regions. Thus, we do not find the contrast between LRR- and TIR-determined specificity to be contradictory. Indeed, these differences may be related to the particular *R* gene/avirulence gene interactions in each case. The avirulence genes that correspond to *L2*, *L6*, and *L11* (for which data indicate LRR-controlled specificity) map to unlinked locations in flax rust (see Lawrence, 1988). However, the corresponding avirulence genes to *L6* and *L7* (for which specificity differences are determined by the TIR region) are tightly linked and have not been separated by recombination. In this regard, it will be of interest to determine whether avirulence genes corresponding to *L10* and *L2–L10Sph*, which have identical LRR regions, are also tightly linked. The question of whether the unlinked avirulence genes encode unrelated products and whether the tightly linked (or allelic) genes, possibly the result of gene duplication events, encode related products will require the cloning of the corresponding avirulence genes from flax rust.

Several chimeric genes had no resistance activity. For example, the *L6–L10Sph* exchange did not confer rust resistance in transgenic plants. This exchange is particularly interesting because three other intragenic exchanges made at the same location were functional. Amino acid differences encoded by the region upstream of the SphI restriction site in *L6* may fail to form a productive interaction with downstream sites in the L10 protein. A similar explanation may account for nonfunctional exchanges in the LRR region. If the LRR regions of L proteins form binding surfaces with multiple ligand contact points, as occurs in the porcine ribonuclease inhibitor protein (Kobe and Deisenhoffer, 1995), then intragenic exchanges between *L* alleles at the NdeI and XhoI sites, which occur between variable regions in the LRR region, would create novel binding surfaces with different properties. Thus, lack of resistance function could be due to the disruption of the putative ligand binding region of the parental L proteins or to the absence of a corresponding ligand in any of the rusts used to assay the chimeric genes.

Alternatively, the combinations of coding region differences in certain chimeric *L* genes may give rise to unstable protein products. Antibodies specific to the chimeric L proteins that do not detect the closely related L9 protein expressed in the host line used for transformation will be required to address the issue of protein stability. Nevertheless, the failure of certain recombinant *L* genes to function may indicate that evolution of new resistance specificities is not a single-step process. Inactive recombinant alleles may result from intragenic recombination; they then act as intermediates that can be "fine-tuned" by mutation or further recombination to provide novel resistance specificities.

In summary, our data indicate a role for the TIR region and LRR region of L proteins in gene-for-gene specificity in the flax–flax rust interaction. The priorities now are to identify products of the flax rust avirulence genes and to characterize the nature of the interaction between these and the corresponding L resistance proteins.

METHODS

Plant Material

Flax lines carrying different *L* alleles (except *L8* and *LH*) were from H. Flor's original set of flax rust differentials and are described by Islam and Mayo (1990). The *L8* allele was from a line in which the *L8* allele of Towner had been backcrossed 14 times into Bison (Flor, 1954). The allele at the *L* locus in Hoshangabad has been designated *LH.* To confirm that the identity of *L3* and *L4* to the *L10* sequence was not due to recent incorrect labeling of lines in our laboratory, we showed independently obtained accessions containing *L3* and *L4* from the USDA flax collection (North Dakota State University, Fargo, ND) to be identical to the authors' original stocks by gel blot analysis of diagnostic polymorphic restriction sites.

Flax Rust Strains

Rust strains H, I, (CH5×I)32, and those with the prefix CH5F2 are described by Lawrence et al. (1981). The strain Sp-y, which recognizes *L2*, was a mutant of CH5F2-133 selected for virulence to *P2*, which allowed it to grow on the Forge line of flax that has resistance genes *L6*, *M*, *N*, and *P2.* The strain WA is a rust isolate collected in Western Australia from Australian native flax, *Linum marginale.* BS-1 (Bison selection 1, virulent to *L9*, avirulent to *L10*) was a segregant from the selfing of strain GFi-1. Strain GFi-1 itself was derived from a cross between WA and a rust from cultivated flax. The additional rust strains that detect *L10* and were used to test chimeric *L10* alleles were strains H, (CH5×I)32, I, and J.

Resistance Gene Cloning

The *L2*, *L10*, and *LH* alleles were cloned in the λ vector EMBL4 (Promega) by using DNA isolated from flax lines homozygous for each allele. Gel blot analysis of EcoRI-restricted DNA using the probe *Lu-1* from the promoter region of *L6* (Lawrence et al., 1995) identified single EcoRI fragments of ~8 to 9 kb. Plant DNA and DNA of the λ vector EMBL4 was digested with EcoRI and ligated. Because genomic DNA gel blot analysis had indicated that no SalI restriction sites occurred within the target EcoRI fragment, the ligation mixes were cut with SalI before packaging to eliminate background clones.

The λ libraries were screened with probe *Lu-1* (Ellis et al., 1995), and the EcoRI inserts were subcloned into the plasmid vector pUC119. The cloned fragments were the same size as the EcoRI fragments observed in genomic DNA gel blots.

The *L*, *L1*, *L3*, *L4*, *L5*, *L7*, *L8*, *L9*, and *L11* alleles were cloned after amplification by using *L* locus–specific long-range polymerase chain reaction (PCR), as described by Anderson et al. (1997). The 5′ primer (5 prime-L) is located 1430 bp upstream of the ATG translation initiation codon and has the following sequence: 5′-TGAG(**GAGCTC**)CGAGAACTTCGAATTCCACAGC-3′. The 3′ primer (3 prime-L), which is located 330 bp downstream of the translation stop codon of the *L* gene, is 5′-GTCC(**GAGCTC**)ATATAACGCTGCTCATCCAC-3′. The sequences matching the *L* gene are underlined. Several mismatches were introduced into the primers to create SacI restriction sites (shown in boldface and enclosed within parentheses) to facilitate cloning of PCR products. For sequence analysis of PCR-generated clones, three clones from two independent amplification reactions were sequenced. DNA sequence compilations and comparisons of DNA and amino acid sequences were performed using the Wisconsin Genetics Computer Group package (version 8; Genetics Computer Group, Madison, WI) and GeneDoc (available at http://www.concentric.net/~Ketchup/genedoc.shtml).

Plant Transformation and *L* Gene Constructs

Transformation of flax was as previously described (Anderson et al., 1997). The *L2* and *L10* alleles used for transformation consisted of the cloned EcoRI fragments isolated from the EMBL4 clones. The 5′ site occurs ~1.4 kb upstream of the ATG codon for translation initiation, and the 3′ EcoRI site occurs ~1 kb downstream of the stop codon. The *L6* clone used in transformation was derived from two overlapping EMBL4 clones (Lawrence et al., 1995) and extended ~5.5 kb upstream of the ATG. In more recent transformations, not reported in this study, a smaller clone, beginning at the same EcoRI site as used in *L2* and *L10* constructs, was used, and this shorter *L6* clone also was fully functional.

The 35S–*L6* cDNA construct was made using the incomplete cDNA clone (Lawrence et al., 1995) to which the missing exon 1 sequence and nontranslated region to a SacI site 163 bp upstream of the ATG was added from the genomic clone of *L6.* The cDNA clone had retained intron 3 (see Lawrence et al. [1995] for details). The cDNA was cloned into an expression vector containing the 35S RNA promoter of cauliflower mosaic virus and the 3′ noncoding region of the nopaline synthase gene.

Chimeric genes were made by exchanging sequences between cloned *L2*, *L6*, and *L10* genes at restriction sites identified by the sequence analysis. All constructs were checked extensively by restriction enzyme site mapping and DNA sequencing over the junctions of the chimeras. In the case of *L6–L10Sph*, which in contrast to the other three intragenic exchanges made at the SphI site did not provide detectable rust resistance in transgenic plants, the complete coding region from the 5′ SacI site to the 3′ BglII site was sequenced using as template the binary vector present in the *Agrobacterium tumefaciens* strain used for transformation.

Transcript Analysis

Specific transcription of the *L6–L10Sph* chimeric gene was detected using the reverse transcriptase–PCR method described by Lawrence et al. (1995). RNA isolated from leaves or tissue-cultured pedicels was reverse transcribed and then amplified by PCR using primers P1 (Lawrence et al., 1995) and P3-10 (5′-CCCCTCCAAGTTGTTTCC-3′) located 680 bp downstream. This involved designing one of the PCR primers to the spliced junction region of exon 2 and exon 3 to prevent amplification of residual genomic DNA in RNA preparations.

Gel Blot Analysis Using Transgenic Plants

The procedure for gel blot analysis has been described in Ellis et al. (1992). The presence of the *L2* allele or chimeric genes containing the 3′ end of *L2* was detected in all genetic backgrounds after EcoRI digestion of flax genomic DNA by using probe *Lu-1* derived from the promoter region of *L6* (Lawrence et al., 1995). The *L2* fragment was substantially longer than were the EcoRI fragments derived from the resident *L* gene alleles in the lines used for transformation due to the presence of two extra direct repeats of ~450 bp in the leucine-rich repeat (LRR) region of this allele. The presence of the *L6* transgene also was detected in EcoRI digests as a 4.1-kb fragment, which is smaller than is the resident *L* allele in the transformed host line due to the presence of a polymorphic EcoRI site in the LRR coding region of *L6.* EcoRI digestion did not reveal a polymorphism between the *L10* transgene, chimeric transgenes involving the 3′ part of *L10*, and the resident *L* alleles present in the lines used for transformation. Therefore, these transgenes were detected with probe *Lu-1* after double digestion of genomic DNA with EcoRI and HindIII or EcoRI and NcoI. The HindIII site and NcoI site are polymorphic sites in *L10.* A polymorphic NcoI site also occurs in *L2* but at a different location than the site in *L10.* The identity of chimeric *L* genes in transgenic flax lines was confirmed by double digestion of DNA with EcoRI, which cuts at a monomorphic EcoRI site upstream of the *L* gene and any enzyme that detects an allele-specific restriction site, such as HindIII in the LRR region of *L10.* The copy number of transgene inserts was estimated by counting the number of "junction fragments" between the right T-DNA border and plant DNA detected using probe *Lu-1* and genomic DNA digested with HindIII. The probe detects fragments resulting from digestion at an HindIII site in the *L* gene sequence and an HindIII site in the plant genome adjacent to the T-DNA insertion.

GenBank Accession Numbers

The GenBank nucleotide sequence database accession numbers for flax *L* alleles described in this study are as follows: *L*, AF093638; *L1*, AF093639; *L10*, AF093640; *L11*, AF093641; *L2*, AF093642; *L3*, AF093643; *L4*, AF093644; *L5*, AF093645; *L7*, AF093646; *L8*, AF093647; *L9*, AF093648; and *LH*, AF093649.

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