
Gregory J. Lawrence, a E. Jean Finnegan, b Michael A. Ayliffe, and Jeffrey G. Ellis

a Commonwealth Scientific and Industrial Research Organisation, Division of Plant Industry, PO. Box 1600, Canberra, ACT, 2601, Australia
b Cooperative Research Centre for Plant Science, PO. Box 475, Canberra, ACT, 2601, Australia

The L6 rust resistance gene from flax was cloned after tagging with the maize transposable element Activator. The gene is predicted to encode two products of 1294 and 705 amino acids that result from alternatively spliced transcripts. The longer product is similar to the products of two other plant disease resistance genes, the tobacco mosaic virus resistance gene N of tobacco and the bacterial resistance gene RPS2 of Arabidopsis. The similarity involves the presence of a nucleotide (ATP/GTP) binding site and several other amino acid motifs of unknown function in the N-terminal half of the polypeptides and a leucine-rich region in the C-terminal half. The truncated product of L6, which lacks most of the leucine-rich C-terminal region, is similar to the truncated product that is predicted from an alternative transcript of the N gene. The L6, N, and RPS2 genes, which control resistance to three widely different pathogen types, are the foundation of a class of plant disease resistance genes that can be referred to as nucleotide binding site/leucine-rich repeat resistance genes.

INTRODUCTION

Two classes of plant disease resistance genes with simple dominant Mendelian inheritance are known. An example of the first class is the Hm7 gene of maize (Johal and Briggs, 1992). Hm7 controls resistance to the fungal pathogen Cochliobolus carbonum race 1 and encodes an enzyme that inactivates a disease symptom-inducing toxin produced by the pathogen. The second class of resistance genes does not encode detoxifying enzymes. This class is composed of the gene-for-gene or pathogen strain-specific resistance genes and controls recognition of the invading pathogen and the triggering of host responses that include activation of host genes encoding anti-pathogen molecules and hypersensitive cell death at the site of pathogen entry (Lamb, 1994). Commonly, plant species contain many different resistance genes of the gene-for-gene class; each confers resistance to some but not all isolates of a particular pathogen species. For example, in flax, 31 strain-specific resistance genes have been identified that control resistance to different isolates of the flax rust fungus (Melampsora lini), and these map to five loci, K, L, M, N, and P (Ellis et al., 1988). Genetic analysis of this system gave rise to the gene-for-gene hypothesis, which is a classic concept in plant pathology that provides the genetic basis for the specific recognition events involved in plant-pathogen interactions (Flor, 1956). Flor demonstrated that for each dominant host plant gene determining resistance, there is a corresponding dominant avirulence gene in the rust controlling pathogenicity. Subsequently, genetic and molecular analyses of many other interactions between plants and their fungal, bacterial, and viral pathogens indicated that gene-for-gene relationships occur commonly in nature (Keen, 1990). The specificity of gene-for-gene interactions suggests that the initial step in plant disease resistance is a recognition event involving either the direct or indirect products of the corresponding host resistance and pathogen avirulence genes. Four resistance genes of the gene-for-gene class have been cloned recently, namely, the Pto bacterial resistance gene of tomato encoding a serine-threonine protein kinase (Martin et al., 1993), the fungal resistance gene Cf-9 from tomato encoding a leucine-rich repeat protein (Jones et al., 1994), the RPS2 bacterial resistance gene of Arabidopsis (Bent et al., 1994; Mindrinos et al., 1994), and the tobacco mosaic virus resistance gene N of tobacco (Whitham et al., 1994). The latter two encode proteins that contain leucine-rich repeats and a nucleotide binding site.

We have previously described a transposon tagging approach for the isolation of rust resistance genes in flax using the maize transposon Activator (Ac) that was introduced into flax by Agrobacterium transformation (Ellis et al., 1992; Lawrence et al., 1993). This report describes the successful outcome of

1 These authors contributed equally to this work.
2 To whom correspondence should be addressed.
Mutant X75 Contains $Ac$ at the $L6$ locus

Mutant X75 contained a newly transposed $Ac$ (referred to hereafter as transposed $Ac$) not present in its parent (Figure 1A). To determine whether transposed $Ac$ mapped to the $L6$ locus, X75 was crossed to the cultivar Birio that carries the $L6$ gene. Two progeny that inherited transposed $Ac$ were identified by DNA gel blot analysis. These two plants, which were heterozygous for $L6$ and an allele for susceptibility (potentially a tagged $L6$ gene), were each crossed to the cultivar Hoshangabad, which

are $^{32}$P-end-labeled HindIII fragments of bacteriophage $\lambda$ ($\lambda$H). A, Accl; B, BamHI.
(B) Maps of genomic clones 4-3 and $\lambda$ 1534-BglI containing the newly transposed $Ac$ in mutant X75 and flanking flax DNA, including probe Lu-1. A, Accl; B, BamH; BgI, BgII; and H, HinfI.
(C) RFLP mapping of the insertion site of transposed $Ac$ in X75 with respect to the $L6$ rust resistance gene in a testcross family of 88 individuals derived from the parents Forge (genotype $L6/L6$) and Hoshangabad (genotype III). The lengths of the polymorphic fragments detected by probe Lu-1 and the testcross progeny classes are given in the table. Rust reaction (R, resistant; S, susceptible; PR, partially resistant) was determined with rust strain CH5-84 that recognizes $L6$. The potentially recombinant $L6$ allele is represented by a question mark.
contains no known rust resistance gene. Thirty-six progeny (18 from each cross) were tested for L6 rust resistance and also scored by gel blot analysis for the presence of transposed Ac. Eighteen progeny were susceptible, and these all carried transposed Ac; the remaining 18 resistant progeny lacked transposed Ac. These data indicate that transposed Ac in mutant X75 maps at or close to the L6 locus.

The insertion site of transposed Ac was also mapped in a different cross using restriction fragment length polymorphisms (RFLPs). A DNA restriction fragment containing transposed Ac and flanking plant DNA was cloned, and a plant DNA fragment, Lu-1 (Figure 1B), which is located 459 bp from the 3' end of Ac, was used as a probe in DNA gel blot analysis. This probe detected a simple fragment pattern on DNA gel blots, and three RFLPs that distinguish the L6 parent Forge from the susceptible parent Hoshangabad were identified in DNA digested with EcoRI, XbaI, or BglII. The segregation of these RFLP markers and L6 rust resistance was analyzed in a testcross family of 88 progeny derived from the Forge and Hoshangabad parents. Complete linkage between L6 and the two RFLP markers from Forge was detected in DNA digested with BglII and EcoRI (Figure 1C). This analysis demonstrated that the Lu-1 DNA fragment was closely linked to L6 and therefore provided further evidence for tight linkage between transposed Ac and the L6 locus. However, one progeny plant, D237, was recombinant for the RFLP markers. This plant was heterozygous for the Forge and Hoshangabad markers detected in DNA digested with EcoRI and BglII but homozygous for the Hoshangabad marker detected in DNA digested with XbaI (Figure 1C). In addition, the recombinant plant had a novel rust resistance phenotype, referred to as partial resistance, that was intermediate between the fully resistant and fully susceptible phenotypes of the parents. The recombinant individual was analyzed in detail, and evidence presented later indicates that recombination had occurred within the L6 gene itself and that the recombinant gene conferred a modified resistance phenotype.

**A Recombination Event near the Ac Insertion Site Alters L6 Expression**

Restriction maps were constructed of the region surrounding the site of recombination in Forge and Hoshangabad and the recombinant D237. DNA from the two parents and the recombinant was digested with various restriction enzymes (single and double digestions), and DNA gel blots were probed with Lu-1. The lengths of restriction fragments that hybridized to the Lu-1 probe were determined and used to construct restriction maps that positioned the polymorphic restriction sites in the parents (Figure 2A). Comparison of the maps of the parents and the recombinant demonstrated that a crossover had occurred within a region of ~3.7 kb bounded by a polymorphic EcoRV site in the Hoshangabad chromosome and a polymorphic EcoRI site in the Forge chromosome. Consequently, the recombinant chromosome in D237 carried the polymorphic

![Figure 2](image-url)
Xbal and EcoRV sites from Hoshangabad (left side of the map in Figure 2A) and the polymorphic EcoRI and BgIII sites derived from Forge (right side of the map in Figure 2A).

The recombinant D237 was unique among the 88 testcross progeny examined in that it alone had a partially resistant phenotype to the rust strain used to identify the presence of L6. D237 was selfed, and the cosegregation of the partial resistance phenotype and the recombinant chromosome, which was detected by the presence of a 4-kb EcoRI fragment using probe Lu-1, was examined among 16 progeny. The data are summarized in Figure 2B. Ten of the 16 progeny were partially resistant with restricted and delayed rust growth. The remaining six progeny were fully susceptible. All susceptible progeny carried only the 8-kb Hoshangabad RFLP marker; all partially resistant progeny were either homozygous or heterozygous for the 4-kb marker from the recombinant chromosome (Figure 2B). Thus, complete linkage was observed between the partial resistance phenotype and the 4-kb L6-linked RFLP marker. This result is consistent with the segregation of a new allele for partial rust resistance at the L locus. Therefore, it is likely that the site of the crossover in one of the chromosomes of D237 lay within the L6 gene and that the recombinant gene derived from this event is responsible for the partially resistant phenotype. The partial resistance phenotype and fully susceptible phenotype are shown in Figure 3.

**Figure 3.** Comparison of the Susceptible Phenotype with the Phenotype of Partial Resistance Conferred by the Recombinant Gene.

One progeny plant is shown from each of the phenotypic classes described in Figure 2. Plants were photographed 16 days after inoculation with rust strain CH5-84. The resistant L6 parent (not shown) allows no rust sporulation. PR, partially resistant; S, susceptible.

**Figure 4.** Analysis of the Spontaneous L6 Mutants X3A and X117.

(A) The two L6 mutants X3A and X117 were identified among F1 progeny derived from a cross between Forge (L6) and Hoshangabad (l, the recessive allele of L6). The regions to which restriction fragment alterations were located in X117 and X3A are bracketed, and the origins of the probes are indicated by bars. The location of transposed Ac in X75 is also shown. B, BamHI; Bg, BgIII; H, HindII; R, EcoRI; S, SacI; X, XbaI.

(B) A comparison of SacI and BamHI double-restricted DNA from mutant X117, its rust-resistant parent (P), and its rust-susceptible parent (H) using the hybridization probe Lu-1, which detected a new 1-kb fragment (labeled new fragment) in X117.

(C) A comparison of EcoRI and XbaI double-restricted DNA from mutant sector X3A, wild-type sector X3B, and the rust-resistant (F) and susceptible (H) parents using hybridization probe Lu-3. The probe detected the loss of a 1-kb fragment (labeled lost fragment) in the mutant. Length markers at right in (B) and (C) are in kilobases and were provided by HindIII-restricted λ DNA (λH).
Two Additional L6 Mutants Contain Small DNA Alterations at the L6 Locus

Two L6 mutants, X3A (Lawrence et al., 1993) and X117 (this study), which were not of the same origin as X75 (no active Ac present in parents) and were not due to Ac insertion or large deletions (J.G. Ellis and E.J. Finnegan, unpublished data), were examined using L6-linked DNA probes. DNA fragment length differences between the mutants and their parents were detected by DNA hybridization analysis using probes from regions adjacent to the site of insertion of Ac in mutant X75. The locations of these alterations and the origins of the probes are shown in Figure 4A.

DNA from mutant X117 and its rust-resistant parent was digested with EcoRI and hybridized with probe Lu-1. The single EcoRI fragment detected in X117 was ~300 bp longer than the corresponding 4-kb fragment in the parent; a similar increase in length was detected in DNA digested with both BamHI and EcoRI (data not shown). This analysis mapped the alteration in X117 to the 1.9-kb EcoRI-BamHI interval. The alteration was subsequently mapped to the 630-bp SacI-BamHI interval by hybridization with probe Lu-1a (Figure 4B). This probe, which hybridized to a small family of fragments, detected a new fragment of ~1 kb in X117 that was absent in both parents. Tranposed Ac in the X75 mutant had inserted within the same SacI-BamHI interval (Figure 1B).

The X3A mutant was isolated as a bisected plant (Lawrence et al., 1993), and consequently DNA from the rust-susceptible mutant sector could be compared with DNA from the wild-type rust-resistant sector (X3B) and also to its rust-resistant parent. DNA gel blot analysis (data not shown) using probe Lu-1 demonstrated that the XbaI fragment in the mutant sector was ~300 bp longer than the corresponding 12-kb fragment in the wild-type sector and parent plant. Similarly, the BglII fragment was ~300 bp longer than the corresponding 7-kb fragment in the wild type. Because no alteration was observed for the 4-kb EcoRI fragment, it was inferred that the alteration in the X3A mutant had occurred in the 1-kb interval between the EcoRI and XbaI restriction sites (Figure 4A). This interpretation was confirmed by DNA gel blot analysis using probe Lu-3 (Figure 4C). Although this probe detected repeated DNA, it is clear that the 1-kb restriction fragment that was present in the wild-type X3B sector and parent is absent in the X3A sector. Thus, in two independent spontaneous L6 mutants, DNA alterations, which were possibly small insertions, were detected within 4 kb of the insertion site of transposed Ac in the X75 mutant.

Reversion to Resistance among the Progeny of Mutant X75 Is Associated with Excision of Ac

DNA from selfed progeny of X75 was digested with HindIII and examined by DNA hybridization using probe Lu-1 to detect plants homozygous for the newly transposed Ac in X75. Homozygous plants contained a 5-kb Ac-plant DNA junction fragment and lacked the corresponding 8-kb fragment present in plants that contained no copies or only one copy of transposed Ac. Four homozygotes were chosen and selfed to provide large numbers of progeny that were screened for rust-resistant revertants. Among 3105 seedlings inoculated with a rust strain that recognizes the L6 resistance specificity, 37 resistant plants were identified. DNA gel blot analysis of 15 of these...
Analysis of the L6 Gene and Detection of Alternative Transcripts

The wild-type L6 gene was isolated from a genomic library made from Forge DNA using Lu-1 as a hybridization probe. One clone, L4, was analyzed in detail, and a 7.62-kb region of the clone was sequenced (Figures 6 and 7). This sequence was compared with the region flanking Ac in the clone L4-3 (Figure 1B). This demonstrated that the two clones contained the same gene and that the Ac insertion in X75 was located as shown in Figure 6A.

A mixture of DNA probes, including Lu-1a and Lu-3, was used to screen a cDNA library derived from RNA extracted from Forge. Nine clones were partially sequenced and compared to the genomic sequence. Only one clone, cDNA 3, was identical to the L6 sequence (Figure 7). The other clones were between 70 and 97% identical to L6 and were probably transcribed from genes related to L6. The clone cDNA 3, containing an insert of 4 kb, was sequenced. When this sequence was aligned with the genomic sequence, two introns were identified (Figure 6A). After removing the introns from the genomic sequence, two long open reading frames of 2.1 and 1.85 kb were detected. The first begins at an ATG codon 310 nucleotides upstream of the first base in cDNA 3, which evidently is not full length. No additional ATG codons occur prior to the first upstream stop codon in the genomic sequence, and no additional upstream exons occur in the 1.5 kb of 5' sequence. The second open reading frame begins near the middle of the cDNA. The sequence of the region near the ATG initiation codon of the second open reading frame was examined for potential introns, and an 82-bp sequence bounded by 5' GT and 3' AG dinucleotides was found. Splicing these 82 nucleotides from the sequence would give rise to a single open reading frame that incorporates most of the two shorter reading frames. This result suggests that cDNA 3 may have resulted from an incompletely processed mRNA.

To test this hypothesis, we used the polymerase chain reaction to amplify transcripts from the L6 gene and to determine whether this putative intron is processed in vivo. Total RNA was isolated from Forge plants and was reverse transcribed using the primer P2. The resulting cDNA was then amplified

abundant product. The relative abundance of the two products, detected by incorporation of 32P-labeled primer and PhosphorImager (Molecular Dynamics, Sunnyvale, CA) analysis, was determined after separation by acrylamide gel electrophoresis.

(C) The predicted major mRNA product resulting from splicing of exons (EX) 1, 2, 3, and 4 contains an open reading frame of 3882 nucleotides. b, bases.
Cloning the L6 Gene for Rust Resistance in Flax

Figure 7. The Nucleotide Sequence of L6 and Predicted Amino Acid Sequences of the Two Transcripts.

The sequence extends from the SacI site to 225 bp downstream of the right-hand BglII site in Figure 6A. cDNA 3 begins at position 477 and ends at 4841. The 29-amino acid extension resulting from the retention and translation of intron 3 is indicated in lowercase letters and is underlined. The P-loop sequence, GGMGIGKT, and kinase-2 domain, LVVLDD, are overlined, the four leucine residues forming a potential leucine zipper are circled, and the stop codons are indicated by dots. The amino acid (aa) and nucleotide (nt) numbering is provided on the left and right of the sequence. The sequence has GenBank accession number U27081.

using the primer pair P1 and P2. Two major products of 471 and 389 bp were amplified (Figure 6B) and sequenced. The 471-bp product, which represented about 25% of the total products, was identical in sequence to the L6 gene and cDNA 3. The 389-bp product, which represented about 75% of the products, was also identical in sequence over most of its length but lacked the predicted 82-bp intron. Therefore, we concluded that the 82-bp sequence is the third intron of L6 and that it is spliced from approximately 75% of the transcripts. Therefore, at least two different mRNA products are derived from the L6 gene, with the major one containing an open reading frame of 3882 bases (Figure 6C).
Analysis of the Predicted Protein Products of L6

The nucleotide sequence of the L6 gene and the encoded amino acid sequence are shown in Figure 7. Translation of the L6 genomic sequence after removal of the three introns predicts a polypeptide of 1294 amino acids. The protein was analyzed using the Kyte and Doolittle (1982) hydrophathy measure and the computer programs ALOM (Klein et al., 1985) and SIGNALASE (N. Mantei, Swiss Federal Institute of Technology, Zurich) for membrane-spanning regions and signal sequences. The N terminus of the protein contains a potential signal peptide (Figure 8A) but no additional extended hydrophobic regions that could provide a membrane-spanning domain.

The protein also contains the sequence motif GMGGIGKT, which begins at residue 265 and fits the consensus of the P-loop ATP/GTP binding site (Saraste et al., 1990). The kinase-2 motif, LVVLDD, which begins at residue 345 of the L6 gene product, is frequently located downstream of the P-loop in ATP/GTP binding proteins (Traut, 1994). The P-loop and kinase-2 motifs form a nucleotide binding site present in many proteins that interact with ATP/GTP. Both these motifs are overlined in Figure 7. Comparison of the protein with the PROSITE database also identified a potential leucine zipper beginning at residue 610 (the four leucine residues forming this consensus are circled in Figure 7).

The C-terminal region beginning at residue 602 contains a high proportion of leucine residues (18%) compared with 9% in the remainder of the polypeptide, and data base searches using the BLAST computer program (Altschul et al., 1990) detected similarities between the leucine-rich region of L6 and a family of leucine-rich repeat proteins that contain multiple tandem repeats of a leucine-rich consensus of ~24 amino acids (Kobe and Deisenhofer, 1994). Such a repeat unit is not evident in L6. However, the L6 peptide contains two direct repeats of 146 and 149 amino acids that contain 20% leucine residues (Figure 8B) and compose ~40% of the leucine-rich region of the L6 gene product.

The predicted product of the transcript that retains intron 3 is identical to the longer product for the first 676 amino acids but contains an extra 29 amino acids at its C terminus not present in the longer product (Figure 8A). These amino acids result from translation of the nucleotide sequence of intron 3. The resulting protein of 705 residues terminates at a TGA codon beginning 6 bp downstream of the 3' intron-exon junction of intron 3. Therefore, the truncated polypeptide contains only 74 amino acids of the leucine-rich region. The first 18 amino acids of the 29-amino acid C-terminal extension was identified by the ALOM computer program as a possible membrane-spanning region. The region is only weakly hydrophobic, and the biological significance of the extension and its short hydrophobic region is unknown at present.

L6 Is a Member of a Multigene Family

As mentioned earlier, screening a cDNA library with L6-derived probes identified nine clones. Partial or complete sequencing
of these clones revealed the existence of at least five different classes of \(L_6\)-related cDNAs. This finding, indicating that \(L_6\) is a member of a multigene family, was confirmed by DNA gel blot analysis using cDNA probes or clones derived from the coding region of the \(L_6\) gene. For example, probes \(Lu-1a\) and \(Lu-3\) detected complex fragment patterns (Figures 4B and 4C). In contrast, probe \(Lu-1\), which lies upstream of the coding region, detected a single fragment associated with \(L_6\) (data not shown). RFLP data reported elsewhere (Ellis et al., 1995) indicated that most of the members of this gene family map to the unlinked and genetically complex rust resistance locus called \(M\), indicating sequence similarities between the \(L\) and \(M\) groups of flax rust resistance genes.

**DISCUSSION**

**Tagging of the \(L_6\) Gene with \(Ac\)**

In summary, evidence that the \(L_6\) gene in mutant X75 has been tagged by \(Ac\) consists of the following: (1) a newly transposed \(Ac\) in X75 mapped to the \(L_6\) locus; (2) a crossover event in the vicinity of the \(Ac\) insertion was associated with an altered resistance phenotype; (3) two other \(L_6\) mutants contained small DNA alterations in the same region of DNA; and (4) reversion to resistance among descendants of mutant X75 was associated with excision of the newly transposed \(Ac\).

**The \(L_6\) Gene Product Resembles the \(N\) and \(RPS2\) Gene Products**

The product of the tagged gene in mutant X75 resembles those of two recently cloned resistance genes: the tobacco mosaic virus resistance gene \(N\) from tobacco and the \(P\). syringae resistance gene \(RPS2\) from Arabidopsis. All three genes, which control resistance to diverse classes of pathogen, share a common structural organization; they encode similar amino acid sequence motifs, including the P-loop and a C-terminal leucine-rich region comprising up to 50% of the molecule. Amino acid similarity between \(L_6\), \(N\), and \(RPS2\) gene products is greatest in the central region (the products of exon 2 of \(L_6\) and \(N\)), which includes the nucleotide binding site that contains at least two motifs involved in nucleotide binding, namely, the P-loop and kinase-2 domain (Traut, 1994). The P-loop motif and at least five other conserved motifs can be identified by amino acid sequence alignments (Figure 9A). This sequence similarity suggests that these genes may function by a similar mechanism, possibly acting in ATP/GTP-dependent transduction of signals encoded by the corresponding pathogen avirulence genes. In light of the similarity between these genes that confer resistance to taxonomically distant pathogens, similar nucleotide binding site/leucine-rich region genes may operate in many gene-for-gene interactions, possibly even interactions between plants and insect and nematode pests.

**The \(L_6\) Gene Product Is More Similar to the \(N\) than to the \(RPS2\) Gene Product**

Several features indicate a close evolutionary relationship between the \(L_6\) and \(N\) genes. This similarity, most striking in the N-terminal half of the polypeptides encoded by exons 1 and 2 (37% identity and 62% similarity; Figure 9B), includes conservation of the locations of introns 1 to 3. The first exon encodes amino acid sequences conserved between the products of \(N\) and \(L_6\) but not those of \(RPS2\). Whitham et al. (1994) have noted similarity between this region of the \(N\) protein and the cytoplasmic domains of the receptor proteins Toll and IL-1R.

The alignment of the products of the \(L_6\) and \(N\) genes (Figure 9B) indicates that the \(L_6\) gene product has an extra 60 amino acids at the N terminus that have no counterparts in the \(N\) gene product. A potential signal peptide occurs in this region of the \(L_6\) gene product (Figure 7A), suggesting that the \(L_6\) gene product enters the secretory pathway. The \(N\) gene product is thought to be cytoplasmic (Whitham et al., 1994). Thus, although the conserved regions of the two gene products suggest a similar mode of action, the products may be active in different subcellular locations.

**The \(L_6\) and \(N\) Gene Transcripts Undergo Alternative Splicing**

Alternative transcripts have been identified for both the \(L_6\) and \(N\) genes (Figure 6; Whitham et al., 1994; Dinesh-Kumar et al., 1995). The alternative transcripts potentially give rise to long and truncated gene products. Both truncated products lack the major part of the leucine-rich region and have a short C-terminal extension of 29 amino acids and 36 amino acids in the products of \(L_6\) and \(N\), respectively.

The truncation of the \(L_6\) gene product is achieved by retention and translation of the 82-bp third intron and termination of translation at a stop codon that occurs in the gene sequence 6 bp downstream from the normal intron 3–exon 4 boundary (Figure 7). The truncation of the \(N\) gene product results from alternative splicing of a 70-bp exon that occurs within intron 3 (Whitham et al., 1994; Dinesh-Kumar et al., 1995). The occurrence of transcripts of both the \(L_6\) and \(N\) genes that in each case involve alternative splicing of the third intron strongly suggests that alternative transcripts are important for the function of both \(L_6\) and \(N\) gene resistance. We are presently attempting to detect the truncated \(L_6\) protein in vivo.

**The Leucine-Rich Regions**

\(L_6\), \(N\), and \(RPS2\) encode a leucine-rich region that involves up to 50% of the gene products. Although the leucine-rich regions of the \(N\) and \(RPS2\) gene products are made up of 14 repeats of ~24 amino acids (Bent et al., 1994; Mindrinos et al., 1994; Whitham et al., 1994), a similar repeat structure is not evident in the \(L_6\) gene product. However, the C terminus of the \(L_6\) gene product contains two direct repeats of 146 and
149 amino acids with 74% identity (Figure 6B). Only a single copy of this repeat is present in the predicted gene product encoded by a related cDNA clone, FC4 (J.G. Ellis and E.J. Finnegan, unpublished results), that is most likely a member of the M rust resistance gene complex (Ellis et al., 1995). Although it is not known whether FC4 functions in rust resistance, the presence of repeat number and sequence between FC4 and L6 may have implications for the control of the specificity of rust resistance gene toward their corresponding fungal avirulence genes. This possibility is currently being tested by analysis of several other rust resistance alleles at the L locus that have different gene-for-gene specificities.

**METHODS**

### Plant Material

Flax (*Linum usitatissimum*) cultivars Birio, which contains L6, and Hoshangabad (no rust resistance genes) and the line Forge that is homozygous for the L6, M, N, and P2 rust resistance genes have been described by Ellis et al. (1992).

### Testcross Analysis

Two different testcross families were used for mapping. The first, which was derived from a cross between plants J457 and Hoshangabad, contained 52 progeny segregating for L6, M, N, and P2 and has been described by Ellis et al. (1992). The second family of 36, segregating for L6, which was derived from a cross between a hybrid of Birio and X27, which was testcrossed to Hoshangabad (Lawrence et al., 1993).

### Transposon Tagging

The procedure for tagging rust resistance genes in flax and identifying newly transposed Activator (Ac) elements by DNA gel blot analysis has been described in detail (Ellis et al., 1992; Lawrence et al., 1993). The progeny were screened for mutations by inoculation with ureidiospores of the flax rust strain CHS-84, which carries the A6 avirulence gene. Rust-susceptible mutant individuals were analyzed for Ac-tagged L6 as described previously (Lawrence et al., 1993).

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**Figure 9.** Comparison of the Amino Acid Sequences Derived from the L6, N, and RPS2 Genes. (A) Alignment of the products of the second exons of L6 and N with the corresponding region of the RPS2 gene product using the computer programs PILEUP and PRETTY (Devereux et al., 1984). Identical and conserved amino acids are in uppercase letters, and nonconserved amino acids are in lowercase letters. The six conserved motifs (marked 1 to 6) are shown by dashed lines above the alignment. (B) BESTFIT (Devereux et al., 1984) comparison of the products of exons 1 and 2 of L6 and N is shown. The conserved positions of interior 1, 2, and 3 in the corresponding genes are indicated by numbered arrowheads, and the P-loop region is marked by lines. The two sequences share 62% similarity and 37% identical amino acids. The vertical bars indicate identical amino acids, and the colons and dots represent similar amino acids. The numbers in (A) and (B) indicate the coordinates of the amino acid residues in the full amino acid sequences.
DNA Hybridization and Restriction Fragment Length
Polymorphism Analysis

These procedures were carried out as described by Lawrence et al. (1993).

Isolation of Genomic Clones

DNA from the mutant X75, which contained at least five copies of Ac, was digested with BamH I and cloned into the bacteriophage \( \lambda \) vector EMBL4. The library was screened using a 3' Ac probe (Ellis et al., 1992), and hybridizing clones that contained inserts extending from the BamHI site of Ac to a BamHI site in flanking plant DNA were purified. The clone (\( \lambda \)-4-3; see Figure 1B) containing the newly transposed Ac contained a 9.5-kb BamH I fragment specific to the X75 mutant (Figure 1A). Subcloning of the \( \lambda \) clone provided the L6 DNA probe Lu-1, a 275-bp HincII fragment located in the 5' untranslated region of L6, which occurs 459 bp upstream of the site of insertion of Ac. DNA of a progency plant of X75, which was homozygous for the transposed Ac, was digested with BglII, which does not cut in Ac, and a clone containing the complete Ac element and flanking plant DNA was identified using probe Lu-1. The wild-type L6 gene was isolated from an EMBL4 library containing size-fractionated Forge DNA partially digested with Sau3A and screened with the Lu-1 probe.

Polymerase Chain Reaction Analysis of the Splicing of Intron 3

Primer P2 (5'-CGCCAACATCAGAAAGGCCG-3'), which lies 67 bp 3' of intron 3, was used to initiate reverse transcription of L6 mRNA. Five micrograms of total leaf RNA was treated with 25 units of reverse transcriptase (New England Biolabs, Beverly, MA) in the presence of 0.125 mM nucleoside triphosphates (Pharmacia) and 20 ng of primer P2 in a final volume of 20 µL. After 1 h of reaction at 37°C, the resultant cDNA was heated to 94°C for 2 min and then treated with 10 µg of RNase A for 15 min at 37°C. Two microliters of cDNA was then amplified in a Corbett FTS1 Thermal Sequencer (Corbett Research, Sydney, Australia) for 35 cycles (94°C for 30 sec, 55°C for 30 sec, and 72°C for 1 min) in a reaction mix containing 50 mM KCl, 10 mM Tris-HCl, pH 8.3, 1.8 mM MgCl₂, 0.125 mM deoxynucleoside triphosphates, 1 unit of Taq DNA polymerase, 10 ng of primer P2, and 10 ng of primer P1 (5'-TGTGTGACAAAAAGGGATC-3'). Primer 1 consisted of the last 14 bases of exon 2 and the first five bases of exon 3 so that the primer would amplify only the cDNA derived from the mRNA from which intron 2 had been spliced and not genomic DNA that could be present in the RNA preparation. The reaction products were resolved by electrophoresis in 1.5% agarose. For sequencing, the reaction products were isolated from an acrylamide gel, reamplified, and sequenced directly using the ABI dye terminator kit (Applied Biosystems Inc, Foster City, CA).

Isolation of cDNA Clones

A cDNA library was constructed using an AMRAD-Pharmacia cDNA cloning kit from poly(A)-enriched RNA isolated from leaf and stem tissue of seedlings of the flax line Forge and screened with a mixture of DNA fragments derived from the L6 coding region.

Sequence Analysis

DNA derived from genomic and cDNA clones was cloned into various plasmid vectors and sequenced using an ABI dye–primer sequencing kit (Applied Biosystems Inc). Sequential ExolI deletions were made with the Pharmacia double-stranded nested deletion kit.

Computer Analysis of DNA and Amino Acid Sequence

The Genetics Computer Group (Madison, WI) programs (Devereux et al., 1984) were used to compile and analyze the DNA sequence information. Data base searches were carried out using the BLAST programs (Altschul et al., 1990), and the ALOM program (Klein et al., 1985) was used in analyzing amino acid sequences for potential peripheral and membrane-spanning regions.

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