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Colloquium Paper

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Contrasting complexity of two rust resistance loci in flax

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ABSTRACT **DNA probes from the *L6* rust resistance gene of flax (*Linum usitatissimum*) hybridize to resistance genes at the unlinked *M* locus, indicating sequence similarities between genes at the two loci. Genetic and molecular data indicate that the *L* locus is simple and contains a single gene with 13 alleles and that the *M* locus is complex and contains a tandem array of genes of similar sequence. Thus the evolution of these two related loci has been different. The consequence of the contrasting structures of the *L* and *M* loci on the evolution of different rust resistance specificities can now be investigated at the molecular level.**

There are numerous examples of plant pathogen interactions where the host plant contains multiple resistance gene specificities which each confer resistance to some but not all isolates of a particular pathogen species. Frequently, the multiple resistance specificities map to only a few genetic loci where they either are alleles of a single gene or are closely linked genes. Well-studied examples include the *Rp1* rust resistance locus at the tip of the short arm of chromosome 10 of maize, the downy mildew resistance loci of lettuce, the *Mla* powdery mildew resistance locus in barley, and the *L* and *M* rust resistance gene loci of flax (1). In some examples, clustering of genes controlling resistance to diverse pathogen species has been observed. For example, resistance to the fungus *Cladosporium fulvum* and the nematode *Meloidogyne incognita* are closely linked in tomato (2), and in wheat resistance genes for rust and powdery mildew are closely linked (3). One explanation for clustering is that the genes have arisen by duplication of an ancestral gene followed by divergence to produce different specificities toward strains of a single pathogen species and even resistance against pathogens from different species and kingdoms. While the generality of this view remains to be demonstrated, the cloning and sequencing of three different resistance genes—the *L6* rust resistance gene of flax, the *RPS2* bacterial resistance gene of *Arabidopsis*, and the tobacco mosaic virus resistance gene *N* of tobacco—have demonstrated that in these three cases, there is some structural and organizational conservation between the gene products that involves the ATP/GTP binding motif, the P loop, in the amino-terminal half of the gene products and a leucine-rich region comprising almost half of the carboxyl-terminal portion of the protein (4–6).

One question concerning resistance gene evolution is why have some groups of resistance genes evolved as tandem arrays and others as multiple allelic series? Genetic evidence for these two situations comes from the analysis of the unlinked *L* and *M* rust resistance loci of flax (*Linum usitatissimum*). Thirteen different rust resistance specificities map at the *L* locus, and extensive attempts to recombine different pairs of *L* specificities in coupling have been unsuccessful (7–9). These results imply that the *L* locus contains a single gene with multiple alleles controlling different resistance specificities. Seven rust resistance specificities map to the *M* locus, which appears to be an array of closely linked genes spanning ≈0.5 map unit. Recombination between different pairs of *M* specificities has been observed and the relative positions of four of the *M* genes have been determined (7, 8).

We have recently cloned the *L6* allele of the *L* locus using the *Ac* tagging strategy described by Lawrence *et al.* (10) and this has provided access into a multiallelic resistance locus. Further, as described in this report, the sequence of *L6* is similar to genes at the complex *M* locus. The structure and evolution of these regions can now be explained.

RESULTS

***L6* and *M* Genes Are Members of the Same Gene Family.** Several different DNA probes from the *L6* gene were used to detect restriction fragment length polymorphism (RFLP) markers that distinguish the flax line "Forge," which carries the *L6, M, N,* and *P2* rust resistance specificities from the cultivar "Hoshangabad," which carries no known rust resistance genes. The joint segregation of these RFLP markers and resistance genes was analyzed in a test-cross family of 52 individuals derived from a cross between these two parents.

Probe LU-2 (Fig. 1), from ≈2 kb upstream of the start of transcription of *L6*, hybridizes to two fragments in most restriction digests (flax is an ancient tetraploid). In *Xba* I-digested Forge and Hoshangabad DNA, both fragments are polymorphic, providing four RFLP markers for analysis (Fig. 1). Joint segregation analysis of the four polymorphic DNA markers and the four rust resistance markers among the progeny of the test-cross demonstrated that the Forge markers *LU-2-1A* and *LU-2-2A* are completely linked to *L6* and *M*, respectively.

Probe LU-4, from the 3′ part of the *L6* gene that could encode a leucine-rich region of the *L6* polypeptide, and other probes from the coding region of *L6* hybridize to complex patterns of restriction fragments, and several restriction enzymes revealed multiple polymorphic fragments between Forge and Hoshangabad. For example, following *Acc* I digestion, four polymorphic fragments from Forge DNA cosegregated with the *M* gene (Fig. 2). In *Xba* I digests, at least four fragments in a complex fragment pattern segregated with *M* and only one with *L6*. With all enzymes analyzed, the pattern emerged of multiple polymorphic fragments linked to *M* and single fragments cosegregating with *L6*. The *L6* gene and the *M* genes thus appear to be members of a family of genes with multiple members mapping to the *M* locus.

No RFLP Markers Detected by *L6* Probes Map to the *N* or *P* Loci. The *N* and *P2* rust resistance genes which are linked (10–20 map units) also segregated in the test-cross family

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Abbreviation: RFLP, restriction fragment length polymorphism.

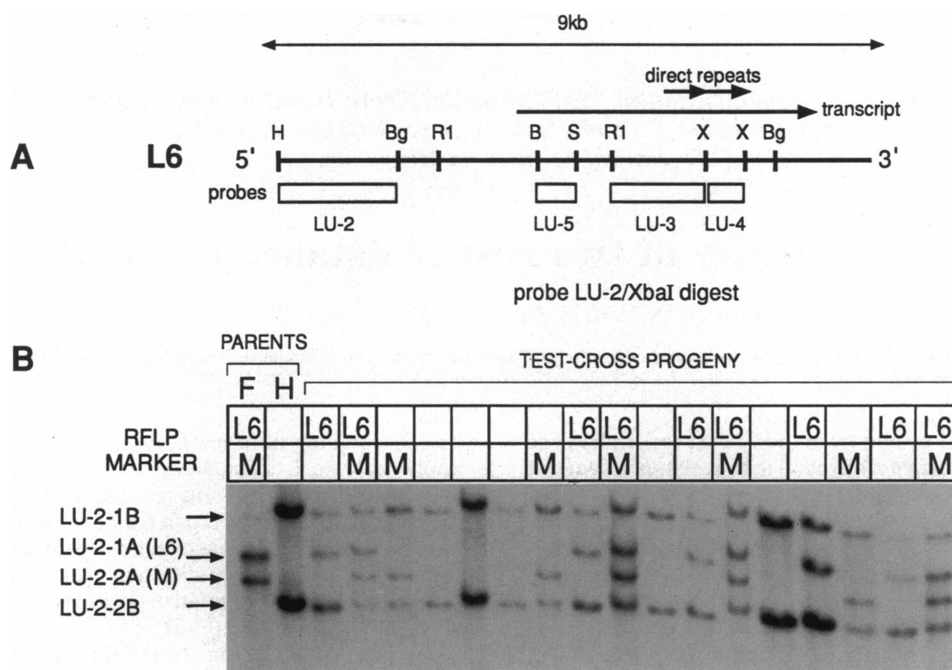


FIG. 1. *L6* gene DNA probes detect RFLP markers linked to the *L* and *M* loci. (*A*) Map of the *L6* region and location of DNA probes. The location of the transcribed region and the two 480-bp direct repeats are indicated with arrows. H, *Hin*dIII; Bg, *Bgl* II; RI, *Eco*RI; B, *Bam*HI; S, *Sph* I; X, *Xba* I. (*B*) RFLP pattern detected in the parents, Forge (F) and Hoshangabad (H), and some of the 52 test-cross progeny using *Xba* I-digested DNA and probe LU-2. The presence of the *L6* and *M* resistance genes was determined by inoculation with the appropriate rust strains. The absence of these genes is indicated by a blank square. The positions of the DNA fragments that provide the four RFLP alleles *LU2-2A/B* (*M* linked) and *LU-2-1A/B* (*L* linked) are indicated. In each case, the *A* allele is derived from Forge and the *B* allele is from Hoshangabad.

described above. Both of these genes are unlinked to *L6* and *M*. No RFLP markers that cosegregated with these loci or with any other locus unlinked to *L6* and *M* were detected with the *L6* probes. While these data are essentially negative, they imply that the *N* and *P* resistance gene loci are not or are only distantly related to the *L* and *M* resistance genes.

Map of the *M* Locus and Analysis of M Mutants. Probe LU-2 and a second probe, X22-1, which is unrelated to *L6* and detects an RFLP marker located 2 map units from the *M* locus, were used to analyze several independent mutants involving the *M* gene that arose either in experiments aimed at tagging *M* with the transposon *Ac* or in control experiments to establish the mutation rate of the gene. Fifteen out of 29 mutants were deletions. For example, mutant plant X22A lacked the *M*-linked DNA marker *X22-1A* and the *M*-linked polymorphic restriction fragments detected with the *L6*-derived probe LU-4. It did however retain the *M*-linked marker *LU-2-2A*. These data indicate that X22A is a deletion mutant and that the order of the markers is as indicated in Fig. 3. A second mutant, X125, possessed the two flanking markers but had lost several but not all *M*-linked polymorphic markers

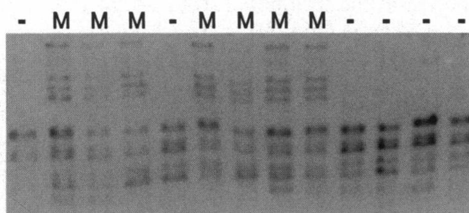
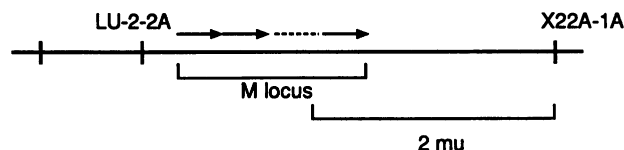


FIG. 2. Probe LU-4 from the coding region of *L6* detects multiple polymorphic fragments linked to *M*. The *Acc* I-digested DNA of several of the 52 test-cross progeny hybridized with LU-4 is shown. The presence of the *M* gene is indicated by *M* and its absence by −.

detected by probe LU-4 and was therefore an interstitial deletion. A third mutant, X107, contained a larger deletion; it had lost all the *M*-linked markers (Fig. 4). These probes that detect flanking and intralocus DNA markers, together with the availability of deletion mutants, provide the means for a detailed molecular analysis of the complex *M* gene region and should assist in cloning the *M* specificity.

DISCUSSION

The molecular analysis of the *L* and *M* rust resistance loci is in complete agreement with the conclusions of earlier genetic analyses (7–9). The *M* locus is complex, consisting of an array of linked genes related in sequence. The evidence is multiple



	LU-2-2A	LU-4 markers	X22A-1A
FORGE	+	+	+
X22A	+	-	-
X107	-	-	-
X125	+	-(*)	+

FIG. 3. Map of the *M* region of Forge. The *LU-2-2A* marker is described in the legend to Fig. 1. The *X22A-1A* marker is detected by DNA probe X22A-1, which was isolated adjacent a T-DNA insert in a transgenic line of Forge that is located 2 map units from *M*. The presence or absence of three Forge RFLP markers linked to *M* in mutants X22A, X107, and X125 is indicated in the table. LU-4 markers are as indicated in Fig. 4. An asterisk (*) signifies that not all of the *M*-linked markers detected by probe LU-4 were deleted in X125.

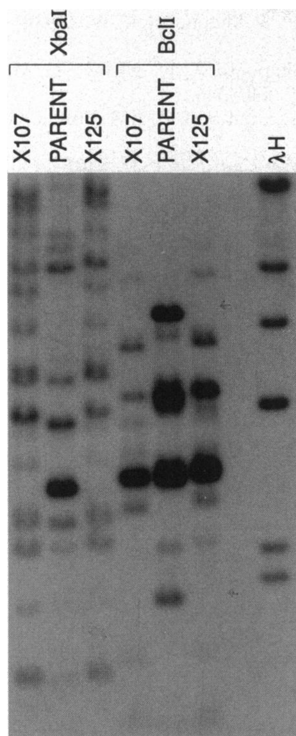


FIG. 4. Restriction fragment patterns observed when DNA from two *M* mutants, X107 and X125, and from their rust resistant parent (a transgenic Forge line) were hybridized with probe LU-4. DNA was digested with *Xba* I and with *Bcl* I. Restriction fragments present in the parent and absent in the mutants are linked to *M* and have been lost together with the *M* gene by deletion. Restriction fragments present in the mutants and absent in the parent are polymorphic fragments derived from the second parent Hoshangabad, which is not included in this figure.

polymorphic fragments detected by probes from the coding region of *L6* that map to the *M* locus. Initial pulsed-field electrophoretic analysis of the *M* locus indicates that the size of the region is between 200 and 1000 kbp (P.A.A., unpublished data). The *L* locus is genetically simple; only single polymorphic fragments map to *L* and cloning and sequencing of the *L6* allele indicate that the gene is ≈5 kb in length.

The parent line Forge contains only a single recognizable rust resistance specificity at the *M* locus. Are the remaining genes detected at this locus by hybridization expressed, and what do they encode? cDNA clones isolated from Forge using *L6* probes fall into at least four distinct sequence species. While none of the cDNAs, except the *L6* cDNA, has been genetically mapped, the fact that *L6* coding region probes identify only the *L* and *M* loci and that only a single gene occurs at the *L* locus implies that the other cDNA clones are transcribed from the *M* region. Therefore it appears that apart from the *M* specificity, other members of the *M* complex are transcribed in Forge. These may represent either the "null" alleles of the remaining six specificities (*M1, M2, . . .M6*) present in other flax lines or resistance specificities that cannot be recognized by the available pathogen isolates.

Flax is an ancient tetraploid and the molecular data described here indicate that the *M* and *L* loci are homoeologous. Why then have the two loci evolved in different fashions with multiple alleles of one gene at the *L* locus and tandemly arrayed resistance genes at the *M* locus? One possibility is that it is the result of chance. A rare duplication event involving a single copy ancestral *M* gene may have occurred. Some repeated DNA structure surrounding the *M* locus but absent from the *L* region may have enhanced duplication of the intervening *M* gene. For example, unequal crossing-over involving two direct copies of a transposable element that flanked the *M* locus could have selectively duplicated the locus. Once duplicated, further unequal crossing-over could then take place, allowing rapid amplification of the locus.

One advantage for breeders of a complex locus is that multiple resistance specificities can be accumulated at the locus in coupling (resistance gene pyramiding). In contrast, in a self-fertilizing species like flax, only single resistance specificities can be accommodated at the *L* locus. The question is whether the advantage of pyramided resistance genes in breeding programs for use in monocultures also applies in natural populations and has this advantage provided the driving force for the evolution of complex resistance loci? Different specificities have been experimentally combined at the *M* locus by selecting recombinant progeny of hybrid plants containing two different *M* specificities. However, naturally occurring examples of two specificities in coupling are rare. In flax, only a single and still unconfirmed example of two *M* specificities occurring naturally in coupling, *M1* and *M4* in the variety "Victory A," has been proposed (11). In wheat, the failure of stem rust to overcome the *Sr26* gene, introgressed from *Agropyron elongatum*, is frequently explained by proposing that the alien gene segment contains multiple linked resistance specificities, but this too is conjecture. It is not clear whether the rarity of naturally occurring as against experimentally produced rust resistance specificities in coupling results from a limited search in nature. The demonstration that two different specificities occur in coupling is not trivial. It requires the appropriate rust stains to distinguish the specificities and the detection of recombinants in a test cross. Alternatively, this situation could be demonstrated by genetic analysis of rust where two avirulence genes segregate that detect resistance specificities at the same locus.

A second potential advantage for tandemly arrayed resistance gene sequences which does not require that all of the copies are expressed and active concerns the evolution of new resistance specificities in the host plant to counter the evolution of parasites to increased host range. Unequal crossing-over within tandem *M* gene sequences, whether from active or silent genes, could provide a mechanism for generating recombinant genes with new resistance specificities. This mechanism has been proposed as a means of generating resistance specificities at the complex *Rp1* rust resistance locus in maize (12, 13) and experiments are underway to test this (S. H. Hulbert, J. L. Bennetzen, and T. Pryor, personal communication).

In the absence of tandemly repeated resistance genes at the *L* locus, an alternative means of generating new allelic specificities must act to account for the 13 *L* alleles that have been identified. One mechanism that may occur has been revealed by sequencing the *L6* allele. The 3′ region of this gene, which encodes a leucine-rich region of the *L6* polypeptide, contains two directly repeated sequences of 480 bp which are 85% identical. Comparison of the *L6* allele to restriction maps of two other alleles indicates that this region may vary in both sequence and repeat number. Therefore, this may be a region of the gene that determines specificity differences between alleles. The repeat structure could permit unequal intragenic recombination leading to amplification or reduction of repeat numbers within a single gene. It is also conceivable that there is information exchange between the unlinked *L* and *M* genes by ectopic recombination.

Although much of this discussion is speculative, it does raise important issues concerning the maintenance and generation of resistance during the coevolution of a host plant and its biotrophic obligate parasite. The availability of cloned rust resistance genes now makes it possible to investigate these questions experimentally.

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