Autoactive Alleles of the Flax *L6* Rust Resistance Gene Induce Non-Race-Specific Rust Resistance Associated with the Hypersensitive Response

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L6 is a nucleotide binding site-leucine rich repeat (NBS-LRR) gene that confers race-specific resistance in flax (*Linum usitatissimum*) to strains of flax rust (*Melampsora lini*) that carry avirulence alleles of the *AvrL567* gene but not to rust strains that carry only the virulence allele. Several mutant and recombinant forms of *L6* were made that altered either the methionine-histidine-aspartate (MHD) motif conserved in the NBS domain of resistance proteins or exchanged the short domain C-terminal to the LRR region that is highly variable among *L* allele products. In transgenic flax some of these alleles are autoactive; they cause a gene dosage-dependent dwarf phenotype and constitutive expression of genes that are markers for the plant defense response. Their effects and penetrance ranged from extreme to mild in their degree of plant stunting, survival, and reproduction. Dwarf plants were also resistant to flax rust strains virulent to wild-type *L6* plants, and this nonspecific resistance was associated with a hypersensitive response (HR) at the site of rust infection. The strongest autoactive allele, expressed in *Arabidopsis* from an ethanol-inducible promoter, gave rise to plant death dependent on the enhanced disease susceptibility 1 (*EDS1*) gene, which indicates that the mutant flax (*Linaceae*) *L6* gene can signal cell death through a defined disease-resistance pathway in a different plant family (*Brassicaceae*).

Additional keywords: autoactive resistance, NB-ARC domain.

Plant disease resistance frequently depends on recognition of invading pathogens and activation of defense mechanisms. Recognition is most commonly determined by a specific plant resistance (*R*) gene and a corresponding avirulence (*Avr*) gene in the pathogen, suggesting that the *R* protein interacts with the product of the *Avr* gene (Ellingboe 1981), although such interactions have been demonstrated experimentally for only three *R/Avr* protein interactions (Deslandes et al. 2003; Jia et al. 2000; Scofield et al. 1996; Tang et al. 1996). Another model is that *R* proteins “guard” or monitor pathogen-induced changes in the presumptive normal cellular targets of *Avr* proteins that have a likely virulence role in the absence of specific host *R* genes (Dangl and Jones 2001; van der Biezen and Jones

1998). At least three cases of *R* proteins guarding host proteins have been documented (Axtell and Staskawicz 2003; Mackey et al. 2003; Shao et al. 2003). In either model, the specific direct or indirect interaction of the *R* and *Avr* proteins activates plant defense responses. About 47 plant disease-resistance genes (i.e., genes with a demonstrated resistance function) have been cloned from different plant species. Of the five distinct classes of resistance protein that have been identified (Dangl and Jones 2001), the largest group (at least 40 genes so far) is the nucleotide binding site-leucine rich repeat (NBS-LRR) class. This class is divided into two groups based on whether the N-terminal domain has a predicted coiled-coil (CC) structure or toll-interleukin-1 (TIR) homology. *L6* from flax, a member of the second group (Lawrence et al. 1995), is the focus of this study.

Three short amino acid sequence motifs, the kinase-1a or P-loop (phosphate-binding loop), kinase-2, and kinase-3 motifs, characteristic of previously defined proteins that hydrolyze ATP or GTP (Traut 1994), occur in the NBS domain of resistance proteins, and hydrolysis of ATP has been demonstrated experimentally for two NBS-LRR proteins, *I2* and *Mi* (Tameling et al. 2002). At least five additional amino acid motifs of unknown function are conserved in the NBS region of NBS-LRR proteins and occur between the kinase-3 motif and C-terminal domain LRR (Collins et al. 1998; Grant et al. 1995; Lawrence et al. 1995). Kinase-1a, kinase-2, and kinase-3 and at least some of the additional *R* protein-conserved motifs are also present in the same relative order in the animal proteins APAF-1 and CED-4, which are involved in regulation of cell death (Chinnaiyan et al. 1997; van der Biezen and Jones 1998), and the central NBS domain of these animal and plant proteins has been referred to as the NB-ARC (nucleotide binding-Apaf1-resistance-CED4) (van der Biezen and Jones 1998) or Ap-ATPase (apoptotic-ATPase) (Aravind et al. 1999) domain. The most C-terminal of the conserved plant resistance gene motifs has been termed motif 5 (van der Biezen and Jones 1998) or the MHD (methionine-histidine-aspartate) domain (Bendahmane et al. 2002; Collins et al. 1998). A similar sequence (LHD) occurs in Apaf-1, but is not clearly evident in CED-4 (van der Biezen and Jones 1998), and is not detected in the mammalian NBS-LRR proteins CARD4, NOD1, or NOD2 (Bertin et al. 1999; Inohara et al. 1999; Ogura et al. 2001).

Activation of resistance proteins in plants by infection with pathogens expressing corresponding avirulence genes commonly induces localized cell death (the hypersensitive response [HR]) as part of the resistance response. This lethal capacity of resistance proteins clearly needs to be tightly regulated in the absence of pathogens. Two current models for this

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regulation, based on strong experimental evidence, involve folding of R proteins into inactive forms through either intramolecular interactions (*cis* repression) (Hwang and Williamson 2003; Moffett et al. 2002) or inhibition of R protein activity via binding of repressor proteins (*trans* repression), for example RIN4 to the RPS2 resistance protein (Axtell and Staskawicz 2003; Mackey et al. 2003). Both forms of repression could act simultaneously in any one resistance protein. The presence of the pathogen avirulence protein is postulated to directly or indirectly activate R proteins through induced conformational changes (Moffett et al. 2002). This could happen by Avr protein-dependent interruption of repressive intramolecular interactions, by releasing or degrading a bound *trans* repressor, or both. These *cis*- and *trans*-repressive interactions can also be disrupted by mutations that lead to constitutive expression of plant defense responses (autoactivity). Instances of autoactivity of various NBS-LRR proteins, that is activity in the absence of a pathogen or avirulence gene product, have so far been described for at least nine point mutations in different domains of several NBS-LRR proteins (Bendahmane et al. 2002; Shirano et al. 2002; Zhang et al. 2003). Autoactivation can also occur through mutation of resistance protein-specific *trans*-acting repressor genes (Axtell and Staskawicz 2003; Mackey et al. 2003; Yang and Hua 2004). A third way to autoactivity of R proteins is through overexpression of R genes (Bendahmane et al. 2002; Grant et al. 2003; Mindrinos et al. 1994; Oldroyd and Staskawicz 1998; Stokes and Richards 2002; Stokes et al. 2002; Tao et al. 2000), which may titrate *trans*-acting repressors or increase beyond an activity threshold the steady-state level of R protein molecules, a proportion of which may exist in a spontaneously active state (Tao et al. 2000). Theoretically, mutations in R proteins that cause substantially increased protein stability could have the same effect.

Using transient expression analysis, Bendahmane and associates (2002) demonstrated that mutation of the MHD motif to MHV in the NBS domain of the virus resistance protein Rx of potato-initiated cell death in the absence of the corresponding avirulence gene product, the coat protein of the *Potato virus X*. This suggested that the MHD motif has a role in repressing defense activation and plant cell death in the absence of the avirulence protein. In the present report, we describe a more extensive analysis, including transgenic plant analysis, of the MHD motif in the flax L6 protein. We also examined the function of a highly variable approximately 20-residue region that occurs C-terminal to the resistance specificity-controlling LRR region in the products of 10 sequenced flax *L* alleles (Ellis et al. 1999), and a role for this region in repressing L6 activity is also demonstrated.

RESULTS

The MHD motif is highly conserved in all plant NBS-LRR proteins.

We examined the amino acid sequences encoded by 40 of the currently known (March 2004) NBS-LRR disease-resistance proteins for conservation of the MHD motif. The motif was identified in all the NBS-LRR proteins (Fig. 1) using the L6 and RPM1 motifs as comparators and The National Center for Biotechnology Information (NCBI) website's Blast 2 program (Altschul et al. 1990) to align sequences. The identification of the MHD motif in each protein was supported by its conserved position relative to the other NBS region conserved motifs. The first three positions, the MHD tripeptide, are highly conserved, the first position being methionine or other aliphatic residues (I, V, L), the second position, histidine, is invariant, and the third position is most frequently aspartic acid. Ten of the 40 proteins, RPS2, Pi-ta, Lr10, RRS1-R, RPP4, RPP5,

RPP11-A, RPP11-B, RPP11-C, and Gro1-4, do not contain the aspartate residue in position 3, and four of these exceptions carry an asparagine residue. The MHD sequence is normally followed in positions 4 and 5 by two aliphatic residues, which are commonly identical in any given protein, while the seventh residue is most frequently negatively charged, with aspartate again being the preferred residue. In some cases among the non-TIR class, the MHD triplet is part of a perfect or imperfect direct repeat, for example Xa1 (MHDxMHD, x is a hydrophobic amino acid) and Hero (VHDxVHD). The sequence alignments also show that positions 9, 10, and 12 are also conserved in the TIR group, and so the MHD motif is presented as a 12-amino acid region in Figure 1.

Some mutants of the MHD motif of L6 induce plant cell necrosis in transient assays.

A set of seven mutants in the MHD motif of the full genomic clone of L6 (M539K, H540A, D541V, N, R, S, and L; henceforth *L6KHD*, *L6MAD*, *L6MHV*, *L6MHN*, *L6MHR*, *L6MHS*, and *L6MHL*; Fig. 2A) was tested in a transient *Agrobacterium* infiltration assay in flax cotyledons and *Nicotiana benthamiana* leaves. The responses of cotyledons of flax line Ward to mutant and wild-type L6 genes are shown in Figure 3A. In Ward, the wild-type L6 gene and the *L6KHD* mutant had no visible effect, the *L6MAD* mutant caused mild necrosis and the *L6MHV* mutant caused severe necrosis that first appeared after about six days and was fully developed by 12 days. *L6MHN*, *L6MHR*, *L6MHS*, and *L6MHL* also induced necrosis in flax although the *L6MHN* mutation had only a mild effect. In *N. benthamiana*, only the *L6MHV* mutation had a clear necrotic phenotype. The reaction took 7 to 14 days to develop in *N. benthamiana*. Because we do not have epitope-tagged alleles or antibodies against the L6 protein, we are unable to determine whether the quantitative differences between the mutant alleles is due to differences in stability between the mutant proteins.

Necrosis was dependent on T-DNA transfer from *Agrobacterium* strains to the plant cell. No necrosis was induced by the MHV mutant when the transformation vector was in an *Agrobacterium* strain that lacked the *vir* genes involved in T-DNA processing and transfer to the plant cell (data not shown). Necrosis in both flax cotyledons and *N. benthamiana* leaves also depended on the wild-type P-loop in L6. A K271M mutation in the P-loop of *L6MHV* did not induce necrosis. The result of the transient assays in *N. benthamiana* leaves is shown in Figure 3B. Necrosis was not induced by the *L6MHV* allele from which the LRR region was deleted (data not shown). So far, we have been unsuccessful in immuno-detection of L6 protein or in expressing epitope-tagged L6 proteins, and so we cannot determine whether the deletion of the LRR has led to protein instability. However, the requirement of the L6 LRR region for necrosis is consistent with previous observations made with the Rx resistance protein in which epitope-tagged and stable Rx MHV mutant protein is inactive when the LRR is removed (Moffett et al. 2002).

All the experiments described above were carried out with the genomic clone of L6 expressed from its own promoter. The assay was also done in flax cotyledons and *N. benthamiana* with the wild-type L6 gene and the D541V mutant expressed using the 35S *Cauliflower mosaic virus* (CaMV) promoter. The mutant and wild-type genes were either derived from a genomic clone (three introns present) or a cDNA clone (no introns). In these experiments, the 35S*L6* MHV gene induced extensive necrosis 1 to 2 days faster than the construct with the L6 promoter. The wild-type 35S*L6* gene also induced some, although much less, necrosis than the mutant but only when the introns were present. This indicates that overexpression of the

wild-type *L6* gene with the strong 35S CaMV promoter can also induce necrosis. This observation is consistent with other reports of the effect of expressing NBS-LRR genes with strong promoters (Bendahmane et al. 2002; Mindrinos et al. 1994; Tao et al. 2000).

Two other *L*-gene variants were shown to induce a necrotic response in transient assays. The first, the domain swap construct *L6-L2H/Bgl* (expressed with the *L6* gene promoter) in

which the variable region of *L* alleles (Ellis et al. 1999) that occurs C-terminal to the LRR domain was exchanged between *L6* and *L2* (Fig. 2B). This construct induced necrosis in *N. tabacum* in transient expression assays (Fig. 3C) and in flax cotyledons (data not shown). An equivalent swap involving *L6* and *L10* (*L6-L10 H/Bgl*) did not induce necrosis. The second necrosis-inducing variant *35SL10 short*, described previously (Frost et al. 2004), expresses a gene that encodes the TIR ho-

Fig. 1. Alignment of the extended methionine-histidine-aspartate (MHD) motif regions in 40 cloned nucleotide binding site-leucine rich repeat gene products with identified resistance activity (as of March 2004). NCBI protein database accession numbers are provided for access to full details and publication references of the genes. All RPP4 paralogs are listed under the same accession number. The RB/Rpi-blb1 genes are the same gene cloned independently. Other genes listed on the same line are orthologues with different resistance specificities. The *Tobacco mosaic virus* resistance protein N is listed as 'N tob' to distinguish it from the flax rust resistance protein N. The proteins have been divided into two groups containing the non-toll interleukin 1 receptor (TIR) class and N-terminal TIR class. Shading indicates the level of conservation at each site (black = 100%, dark gray >80%, light gray > 60%) with amino acid residues of similar chemical properties (aliphatic, aromatic, polar, basic, acidic) considered together.

mology region and 17 amino acids of the NBS region of *L10* and induced a strong necrotic reaction in tobacco leaves (Frost et al. 2004) and in the flax cotyledon assay in the present study (data not shown).

Analysis of autoactive alleles of *L6* in transgenic flax.

The *L6KHD*, *L6MAD*, and *L6MHV* mutant alleles and the *L6-L2H/Bgl* and *L6-L10H/Bgl* domain swaps were tested in transgenic flax. All these constructs contained the *L6* promoter. Ten transgenic plants containing the *L6KHD* mutant allele and their selfed progeny had normal morphology and were susceptible to infection with a rust strain avirulent to *L6*. Although reverse transcriptase-polymerase chain reaction (RT-PCR) analysis indicated that the mutant genes were expressed (data not shown), the encoded protein is either nonfunctional or unstable.

Six of seven *L6MAD* T0 transgenic plants had a dwarf phenotype and their T1 progeny segregated for normal, dwarf, and extreme dwarf plants. The dwarf phenotypes were identical to those shown in Figure 4A and were associated with decreased internode lengths (Fig. 4B and C). Analysis of T2 progeny families from these plants demonstrated that the T1 dwarfs and extreme dwarfs were hemizygous and homozygous, respectively, for the dwarfing gene. T1 progeny were tested for reaction to a rust strain avirulent to *L6*. In the analysis of rust resistance of the T1 progeny families (59 seedlings) there was a complete association between rust resistance and the dwarf phenotypes, which indicated that the dwarf phenotype and resistance were caused by the *L6MAD* transgene. For example, nine progeny of the T0 plant PH253-3 segregated two wild-type (rust susceptible), four dwarf (resistant), and three extreme dwarf (resistant) seedlings. In a second experiment, a further 10 progeny of the T0 plants were tested with a rust strain virulent to *L6*. Wild-type segregants were susceptible and dwarfs and extreme dwarfs were resistant. The resistance reaction of dwarfs to *L6*-avirulent and *L6*-virulent strains could be distin-

guished. Reaction to the former rust involved HR and no rust sporulation, whereas reaction to the latter was tiny sporulating rust pustules on young leaves and complete resistance with HR on older leaves (Fig. 4F). The extreme dwarfs were completely resistant. No spontaneous HR was observed in noninoculated dwarf plants (Fig. 4E). Either the *L6MAD* mutant has gained a new resistance specificity (toward a rust strain that does not possess avirulence to *L6*) or the observed resistance is not due to normal gene-for-gene recognition. Data below suggest that rust resistance to *L6*-virulent rusts is most likely due to autoactivity of the mutant *L6* allele giving rise to constitutively expressed defense responses. Images of the dwarf phenotypes and rust reactions of plants transgenic for autoactive *L6* alleles, either *L6MAD* or *L6-L2/Hind/Bgl* (discussed below), are shown in Figure 4.

We had difficulty in recovering transgenic plants carrying the *L6MHV* allele that was the most active in induction of cell death in transient assays. Only five transgenic plants were recovered from multiple transformation experiments. Three had a normal phenotype, were susceptible to rust, and possibly did not express the mutant gene. Two had a dwarf phenotype in the T0 generation that was more extreme than any *L6MAD* transgenics or their progeny. These T0 plants became chlorotic during growth in soil and were maintained as cuttings, many of which died due to severe leaf necrosis spreading upward from the base of the plant before seed production. Five progeny recovered from a single seed capsule segregated one normal, three dwarfs, and one extreme dwarf that died before flowering. None of the *L6MHV* progeny have been tested for rust reaction because of their extreme phenotype and poor growth.

Seven of eight T0 *L6-L2H/Bgl* transgenics were resistant to a rust strain that is avirulent to *L6* and expressed no obvious abnormal growth phenotypes. Progeny of three rust-resistant transgenics had a wild-type phenotype, while the four others segregated for a dwarf phenotype. These four carried a single transgene insert (DNA gel blot analysis, data not shown).

Fig. 2. A, Mutations introduced by site-directed mutagenesis into the methionine-histidine-aspartate (MHD) motif of *L6*. The mutations marked by an asterisk were tested in both transient assays and in transgenic flax. **B,** The amino acid sequences of the C-terminus of *L6* protein and *L6-L2 H/Bgl* and *L6-L10 H/Bgl* domain swaps that replace the C-terminal polymorphic residues of *L6* with those of *L2* and *L10*. The position of the *Hind*III restriction enzyme cleavage site in the corresponding DNA sequence and used for construction of the recombinants is shown. The *Bgl*II restriction site used for making the exchanges occurs 3' of the stop codon of each allele.

Progeny from three of these four transgenics segregated consistent with a 3:1 ratio (e.g., T0 plant JE3170, discussed below, 46 wild-type and 18 dwarfs—dwarfs were homozygous for *L6-L2H/Bgl*). The fourth family (from T0 plant JE3192) segregated for 15 wild-type, 35 dwarf, and 18 extreme dwarf, consistent with a 1:2:1 ratio in which individuals hemizygous for the transgene are dwarf and those homozygous for the transgene have an extreme dwarf phenotype. This was similar to *L6MAD* mutants. The fact that progeny from three of the seven T0 *L6*-expressing transgenics did not segregate for the dwarf phenotype and only one of the four remaining families segregated for the extreme dwarf phenotype indicates that the *L6-L2H/Bgl* allele is a weaker autoactive allele than the *L6MAD* and *L6MHV* mutants in stable transgenics. Primary transgenic plants expressing the domain swap allele *L6-L10H/Bgl* did not express rust resistance and had no unusual growth phenotypes.

To test the rust-resistance specificity of the *L6-L2H/Bgl* transgene and to confirm the association of the transgene with the dwarf phenotype, seven to ten progeny groups from two primary transgenics (JE3170 and JE3192) were each tested with several rust strains either avirulent or virulent to *L6* plants. In tests with *L6*-avirulent rust strains, the JE3170 fami-

lies segregated 19 wild-type plants (14 rust resistant, 5 susceptible) to 8 dwarf plants (all resistant), consistent with the dwarf plants being homozygous for the transgene and with the phenotypically wild-type plants being either hemizygous for the transgene (and resistant) or lacking the transgene (susceptible) in a 2:1 ratio. With *L6*-virulent rusts, this family segregated 21 normal (all rust susceptible) to 6 dwarf (all resistant). This result indicated that the resistant wild-type plants expressed the *L6* specificity, whereas the dwarf plants, like *L6MAD* dwarfs, expressed a nonspecific resistance. In contrast to the normal *L6* rust-resistance phenotype with no pathogen development, the resistance of the dwarf plants to *L6*-virulent strains was slightly “leaky” in that the younger inoculated leaves produced small uredospore pustules surrounded by necrosis. The other older leaves produced hypersensitive flecks and no apparent rust growth. Rust reactions of *L6-L2H/Bgl* leaves are shown in Figure 4. The rust reactions of *L6-L2H/Bgl* plants are very similar to those of *L6MAD* plants.

Families from JE3192, tested with an *L6*-avirulent rust, segregated 6 normal (all susceptible), 12 dwarfs (all resistant), and 7 extreme dwarfs (all resistant), which also demonstrated a clear association between the presence of the resistance-con-

Fig. 3. **A**, Reaction of flax cotyledons to infiltration with *Agrobacterium* strains containing the ‘empty’ binary vector and the same vector carrying the indicated *L6MHD* mutants. **B**, A leaf of *N. benthamiana* infiltrated with *L6MHV* (bottom) and *L6MHV* with a mutated P-loop (top). **C**, A leaf of *N. tabacum* infiltrated with *L6-L2H/Bgl* (bottom) and the empty vector (top). Infiltration with wild-type *L6* gave reactions very similar to the empty vector (not shown).

ferring transgene and a dwarf phenotype. When tested with *L6*-virulent rusts, progeny segregated 6 normal (all susceptible), 18 dwarf (all resistant), and 9 extreme dwarf (all resistant).

Basis of nonspecific rust resistance.

The rust strains used in this analysis were selfed progeny of strain CH5 (Lawrence et al 1981). Avirulent strains carry two linked avirulence genes, *AvrL567-a* and *AvrL567-b*, that are recognized by *L6*, whereas virulent strains are homozygous for the polymorphic, expressed-but-unrecognized gene *AvrL567-c*, and these genes encode very similar proteins (Dodds et al. 2004). We tested whether the *L6MAD* and *L6-L2Hind/Bgl* autoactive alleles may have gained recognition for the *AvrL567-c* gene product in the *L6*-virulent strains. Using a transient *Agrobacterium* infiltration assay (Dodds et al. 2004), we introduced a control empty vector construct, *AvrL567-a*, expressed from the 35S promoter, and *AvrL567-c*, expressed

from the same promoter, into *L6*, *L6MAD*, *L6-L2Hind/Bgl*, and rust-susceptible Ward flax plants. No leaf necrosis was induced in the susceptible Ward plants. Necrosis was induced by *AvrL567-a* but not by *AvrL567-c* in *L6* leaves, consistent with a specific gene-for-gene interaction. A similar result was observed for the *L6-L2H/Bgl* dwarf plants, although the necrotic reaction to *AvrL567-a* was slower to develop and less extensive, which also indicated *L6* specificity. For the *L6MAD* plants, no necrosis was induced by either *AvrL567-a* or *AvrL567-c*. The weaker response to *AvrL567-a* by the autoactive *L6-L2 H/Bgl* plants, which express *L6* specificity, may be due to increased resistance to *Agrobacterium* infection due to expression of defense responses in these plants (discussed below). The failure of *L6MAD* plants to recognize *AvrL567-a* may be due to either the effects of the defense gene expression on *Agrobacterium* growth, the loss of *L6* specificity by the mutant, or both. The former possibility at least is supported by the

Fig. 4. A, T1 progeny of a transgenic flax plant JE3192 that carried a single copy of the *L6-L2H/Bgl* transgene. Progeny segregate for wild type (plant 1, transgene null), dwarf (plant 2, hemizygote), and extreme dwarf (plant 3, transgene homozygote) phenotypes. The phenotypes of *L6MAD* segregants are identical, while the dwarf and extreme dwarf segregants of *L6MHV* transgenics are more extreme. B and C, Similar length stem segments of dwarf (B) and wild-type (C) T1 segregants derived from a plant transgenic for the *L6MAD* mutant allele. Dwarf plants have reduced internode lengths. D, Leaves 1, 2, and 3 are from a dwarf segregant derived from transgenic plant JE3912 and have been photographed approximately 14 days after inoculation with rust strain CH5F2-78 that is virulent to plants carrying *L6*. Leaves 1 and 2 are younger and show some small pustules, while the oldest leaf 3 showed hypersensitive flecks. Leaf 4 is derived from a control infection of a plant with no effective rust resistance gene. E and F, The detection of autofluorescence associated with the hypersensitive response reaction in uninfected mock-inoculated and rust-infected leaves, respectively, from progeny plants transgenic for the *L6MAD* allele. The rust strain used was virulent to *L6*. The leaves were cleared and viewed under a microscope with UV source to detect autofluorescence and were photographed five days after rust infection. No autofluorescence was seen in compatible rust interactions with wild-type plants (data not shown). G, Transgenic *Arabidopsis* (approximately 4 weeks after germination) carrying *alcA-L6* (left) and *alcA-L6 MHV* (right) were sprayed with 2% ethanol and were photographed five days after treatment.

observation of much-reduced transient-expression β -glucuronidase (GUS) activity in *L6MAD* plants compared with *L6* plants when infiltrated with an *Agrobacterium* strain with a T-DNA carrying 35SGUS.

In *Arabidopsis*, mutants that constitutively express suites of defense proteins, including PR1 and chitinase, frequently have stunted phenotypes (Bowling et al. 1997; Grant et al. 2003; Shah et al. 1999; Shirano et al. 2003; Zhang et al. 2003). We examined some of the dwarf flax lines for constitutive expression of PR1 mRNA and chitinase enzyme activity. T1 progeny of the *L6MAD* transgenic plant PH253-3, which, as shown by DNA gel bot analysis, contained a single transgene insert, were grown in soil, were scored for dwarf phenotype, and

were used for RNA preparation. Two wild-type, three dwarf, and three extreme dwarf progeny (nontransgenic, heterozygous, and homozygous, respectively, for the transgene, as determined subsequently by T2 family analysis) were examined by RNA gel blot analysis of the PR1 gene (Fig. 5A). Very low levels of PR1 expression were detected in the wild-type segregants (transgene nulls), and increased levels of PR1 message were detected in the dwarf (transgene heterozygotes) and extreme dwarf (homozygous) progeny. Expression levels of PR1 in the transgene hemizygotes and homozygotes were approximately 10- and 100-fold higher than the nontransgenics, respectively, indicating an amplification of the response by the increase from one to two transgene copies.

Fig. 5. Pathogenesis-related (PR) gene and chitinase expression in autoactive L6 flax. **A**, T1 segregants derived from selfing a transgenic plant PH253-3 carrying a single copy of *L6MAD* allele. Equal amounts (optical density at 260 nm units) of RNA extracted from two wild-type (W) segregants, three dwarf (D), and three extreme dwarf segregants (E) were probed with a flax PR1 gene probe. The ethidium bromide-stained gel provides the loading control. **B**, Equal amounts of RNA from wild-type (W) and dwarf segregants (D) of two transgenic flax plants carrying the *L6-L2H/Bgl* allele and from wild-type plants sprayed with benzothiadiazole-7-carbothioic-acid-*S*-methyl ester (BTH) were probed with a flax PR1 gene probe. The extreme dwarf progeny from JE3192 were not examined in this experiment, and JE3170 segregated for only wild-type and dwarf progeny. **C**, Chitinase activities were determined for segregating progeny from four different *L6-L2H/Bgl* plants. Plants were classified as dwarf (d) or wild-type (n) by visual inspection and rust resistant (R) or susceptible (S) by inoculation of individual plants with flax rust after tissue samples had been taken for chitinase assays. Extreme dwarf progeny of JE3192 were not examined. Within JE3125, JE3170, JE3192, and JE3164, chitinase activities marked with different letters are significantly different (*t*-test, 95% confidence).

T1 dwarf and wild-type progeny of the *L6-L2H/Bgl* plants JE3192 and JE3170 were examined for PR1 expression by RNA gel blot analysis (Fig. 5B). For JE3192 progeny, a small increase in the level of PR1 expression was observed in dwarfs. RNA was not isolated from the extreme dwarfs (probable transgene homozygotes) that segregated in the JE3192 family. Higher levels of PR1 expression occurred in the JE3170 dwarf progeny (presumed transgene homozygotes), and this expression was equivalent to the level induced in wild-type flax seedlings by spraying with 0.16 mM 1,2,3-benzothiadiazole-7-carbothioic-acid-*S*-methyl ester (BTH) (Fig. 5B).

Progeny of four different *L6-L2H/Bgl* primary transgenics were tested for chitinase enzyme activity (Fig. 5C). In this experiment, we measured chitinase activity in each progeny seedling and then determined the presence or absence of the *L6-L2 H/Bgl* transgene in that seedling by testing its reaction to a rust strain avirulent to *L6* (Fig. 5C, R or S). The dwarf progeny expressed at least fourfold more chitinase activity than the nontransgenic normal progeny. The heterozygous nondwarf progeny expressed a small increase in chitinase expression over the nontransgenic controls (Fig. 5C). Only the heterozygous dwarf and not the extreme dwarf progeny of JE3192 were tested.

Based on the observed increase in PR1 gene expression and chitinase activity in the dwarf plants, we postulate that the increased spectrum of resistance of these transgenics to rust strains normally virulent to *L6* plants is probably not due to classic resistance gene-avirulence gene interaction but rather to autoactivation of plant defenses (i.e., activity of the R protein in the absence of a corresponding Avr protein).

Expression of autoactive alleles using the flax *Fis1* promoter.

Nonspecific resistance and plant stunting are associated with the autoactive *L6* alleles. We attempted (unsuccessfully) to uncouple nonspecific resistance and dwarfing by using a rust-inducible promoter to control the expression of the autoactive alleles. The flax *Fis1* promoter, which is induced around developing rust pustules (Ayliffe et al. 2002), was used to express the *L6/L2 H/Bgl* and *L6MHV* alleles in transgenic flax. Ten *Fis1-L6/L2 H/Bgl* transgenics were recovered. The T0 and T1 plants had a wild-type (not dwarf) phenotype, suggesting that the expression of the *L6/L2H/Bgl* transgene from the *Fis1* promoter may be less than from the *L6* promoter, which is associated with a dwarf phenotype. No nonspecific rust resistance was achieved with this transgene; T1 progeny plants segregated for resistance to a rust strain avirulent to *L6* and were fully susceptible to a strain virulent to *L6*. The *Fis1-L6MHV* transgene behaved differently. Transgenic plants had a dwarf phenotype and expressed nonspecific rust resistance. We conclude that *L6MHV* is a stronger autoactive allele than *L6/L2Hind/Bgl* and that the background (uninduced) level of expression from the *Fis1* promoter (Ayliffe et al. 2002) is sufficient for expression of the dwarf phenotype associated with the *L6MHV*.

Expression of autoactive alleles using an ethanol-induced promoter.

The concept of regulated expression of an autoactive *L6* allele was further explored in both *Arabidopsis thaliana* and flax, using the ethanol-induced *alcA/alcR* promoter/activator system (Caddick et al. 1998). For *Arabidopsis*, 21 *alcA-L6* and 23 *alcA-L6MHV* plants were recovered in the background of the Col-0 ecotype. Their transgenic progeny, which had a wild-type appearance, were treated with 2% ethanol. Progeny of 18 of the 23 *alcA-L6MHV* plants became necrotic within two to four days of treatment, whereas none of the *alcA-L6* plants

showed any effects (Fig. 4G). RNA gel blot analysis indicated that *L6* mRNA could be detected at 3 h after a single ethanol treatment, with peak expression around 6 h posttreatment in both classes of transgenic plant. The expression of the *alcA-L6MHV* gene is shown in Figure 6A. In the *alcA-L6MHV* plants, PR1 gene expression was detected by 24 h (Fig. 6B).

The *alcA-L6MHV* allele was also tested in ecotype WS-0 and the WS-0 mutant *eds1-5* (Falk et al. 1999). Progeny of five of seven WS lines died after ethanol treatment. None of the progeny of 10 transgenic *eds1-5* plants died after ethanol treatment, although four lines showed distinct yellowing. In summary, in both the Col-1 and WS-0 backgrounds, ethanol induced complete necrosis of plants transformed with the *alcA-L6MHV* allele but had no effect on plants transformed with *alcA-L6*. The phenotype was dependent on *EDS1*, although some yellowing was evident in plants carrying *eds1-5*, which is a weak allele of *EDS1* (Falk et al. 1999).

Flax was also transformed with the same two ethanol-regulated constructs. Eight *alcA-L6* transgenics were recovered. Among the seven T0 plants tested, all were susceptible to an *L6*-avirulent rust, which indicated no or insufficient expression of the *L6* gene to give resistance. T1 progeny from the eight T0 *alcA-L6* plants and controls were treated with ethanol. Treatment had no effect on nontransgenic controls. In contrast to the results in *Arabidopsis*, seven of the eight *alcA-L6* T1 flax families segregated for ethanol-induced necrosis that was visible from 12 h after treatment. Six T0 transgenic flax plants carrying the *alcA-L6MHV* allele were recovered. The T1 progeny had a wild-type appearance, and only one of the T0 plants produced progeny that segregated for ethanol-induced necrosis that was more rapid and extensive than *AlcA-L6* plants.

The difference between experiments with flax and *Arabidopsis* may be partly due to the transformation method. Transformation of flax involved a callus stage in tissue culture that can induce the anaerobic response that produces ethanol and acetaldehyde (Dolferus et al. 1985), both strong inducers of

Fig. 6. Ethanol-induced expression of *L6MHV* and *PR1* RNA by ethanol treatment in bulked transgenic progeny derived by selfing an *Arabidopsis* plant transgenic for *alcA-L6MHV*. RNA was isolated from seedlings at the indicated times after treatment, and two gel blots were made. **A**, Results using the *L6* gene probe. **B**, Results using the *PR1* gene probe. The RNA loading controls shown in A and B were provided by reprobing both filters with an 18S RNA probe.

the *alcA* promoter (Junker et al. 2003). Tissue culture induced expression of the strongly autoactive *alcA-L6MHV* would counter-select transformed cells in the callus. The activity of the *alcA-L6* in flax but not *Arabidopsis* transformants could be due to more effective interaction of the L6 protein with presumptive downstream signaling components in flax as compared with *Arabidopsis*.

DISCUSSION

Autoactive *L6* mutants.

Several different autoactive alleles of *L6* have been described here. The autoactive domain-swap allele *L6-L2H/Bgl* is reminiscent of the similar effect of N-terminal domain swaps between paralogous *Mi* genes in tomato, which disrupt intramolecular interactions that normally repress activity (Hwang and Williamson 2003), and also reminiscent of “lesion mimic” recombinants between paralogues of the maize *Rp1* genes (Sun et al. 2001). The variable C-terminal domains of L proteins may interact with upstream variable domains in the L protein and repress activity in the absence of avirulent rust pathogens. Interestingly, while *L6-L2H/Bgl* has an autoactive phenotype, another domain swap, *L6-L2 Sph*, with 5' *L6* and 3' *L2* sequences (Ellis et al. 1999), recombined at a site in the NBS domain, expresses a wild-type *L2* resistance and no obvious morphological phenotype. This exchange site may define an N-terminal limit of the upstream site with which the C-terminal domain may interact.

In the present study of *L6*, we have also extended the earlier observation, made with the Rx resistance protein (Bendahmane et al. 2002; Moffett et al. 2002), that the conserved MHD motif functions as a negative regulator of resistance protein activity. Specifically, we have looked at a larger set of MHD motif mutants and have examined some of these in transgenic plants. The crucial role played by the aspartate residue of the MHD motif of *L6* in repression of the R protein was demonstrated by mutation of this amino acid. Substitution of the negatively charged aspartate with an uncharged hydrophobic aliphatic residue (valine and leucine), a positively charged residue (arginine), a small uncharged polar residue (serine), and by the similar asparagine all induced autoactivity. Substitution of aspartate with glutamate that is also negatively charged was not examined in this study; however, we note that in the many predicted NBS-LRR proteins encoded by the *Arabidopsis* genome, most of unknown function, none carry a glutamate at this position of the MHD motif, suggesting that it cannot functionally substitute for aspartate. The fact that five different substitutions had a similar effect indicates that this conserved motif may be part of a molecular switch and that interaction of the negatively charged aspartate residue with one or more other as-yet-undefined R protein residues locks the activity of the protein in the “off” position. Such a situation is known for a β 2-adrenergic receptor in which arg-131 in a conserved asp-arg-tyr triad is involved in an ionic interaction with the proximal asp-130 and distal glu-268 residue to lock the receptor into the inactive state. This intramolecular interaction was indicated by data from site-directed mutagenesis of the critical residues that cause receptor activation and was further supported by crystallographic analysis of the receptor that shows juxtaposition of the postulated interacting residues in the folded inactive receptor (Ballesteros et al. 2001). A crystallographic analysis of the NB-ARC domain will help to fully elucidate the action of the MHD motif in R proteins. It is interesting to note that, among the 40 cloned R proteins (Fig. 1), 10 do not carry the aspartate residue and 4 (including orthologues RPP4 and 5) carry asparagine, the substitution that has a mild but detectable necrosis-inducing effect on *L6* in transient assays. The effects of the

substitutions in the six other exceptions (D to H, T, Q, and K in Pi-Ta, RPP1-A/B/C, Gro1-4, and RRS1-R, respectively) have not been tested in the context of *L6* or Rx. It may be that the potential autoactivating effects of these exceptions are “compensated” by other sequence differences in the respective R proteins. Mutation of the completely conserved histidine residue (Fig. 1) to alanine (*L6MAD*) had a similar but less drastic autoactive effect than *L6MHV*, as judged by the transient and stable transformation phenotypes. Mutation of methionine to lysine (the *L6KHD* allele) did not cause autoactivation but destroyed *L6* activity, either because the M residue is needed for *L6* activity or because the substitution destabilizes the protein.

The MHD motif has also been identified by sequence similarity and location in the mammalian cell death-control protein Apaf-1 and occurs as LHDQVD and is also conserved in the very closely related proteins from zebra and puffer fish. However, no obvious equivalent sequence occurs in the Apaf-1 homologue DARK in *Drosophila melanogaster* (Rodriguez et al. 1999). As far we are aware, no mutations in the potential MHD-like motif of Apaf-1 have been analyzed to determine whether this sequence is indeed a functional homologue of the plant resistance-protein MHD motif.

For both Rx and *L6*, autoactivity of mutants in the MHD motif is dependent on the wild-type P-loop and LRR region (Bendahmane et al. 2002; Moffett et al. 2002; this study), indicating that ATP binding (and perhaps hydrolysis) and the LRR region are necessary for activity. However, studies by Frost and associates (2004) and this study indicate that expression of a truncated *L10* gene, a closely related allele of *L6*, encoding essentially only the TIR N-terminal region also induces necrosis in transient assays in flax and tobacco and severe autoactive resistance phenotypes in transgenic tobacco. This result is consistent with the TIR region being the major signaling domain of the TIR-NBS-LRR class of resistance proteins and with this signaling capacity being normally repressed by interactions with the remainder of the wild-type L protein. Other work (Bendahmane et al. 2002; Hwang and Williamson 2003; Moffett et al. 2002) similarly indicates a signaling role of the N-terminal CC domain of the CC-NBS-LRR class of resistance proteins and the involvement of intramolecular interactions in repressing signaling. Thus derepression of L rust-resistance proteins can be mediated by i) a cognate Avr protein, ii) by mutations, like *L6MHV* in a P-loop and LRR-dependent fashion, iii) sequence changes in the variable C-terminal variable region, and iv) by deletion of the repressing NBS and LRR domains. The data suggest R proteins are held inactive by intramolecular interactions involving regions including the LRR and MHD and that activation triggered by Avr proteins releases the TIR domain for active signaling.

We have also shown that the autoactive *L6* gene induces a cell death response in transient assays in tobacco species and also when expressed from a regulated promoter in transgenic *Arabidopsis*. *L6* autoactivity in transgenic *Arabidopsis*, like activity of *Arabidopsis* TIR-NBS-LRR genes, is dependent on the *EDS1* gene. Additionally, we have recently demonstrated that the *L6* gene interacts in a gene-for-gene manner with its corresponding flax rust Avr gene in transient assays in tobacco (Dodds et al., 2004). Together, these data show that some resistance genes can function in different plant families.

Do autoactive *L6* alleles retain *L6* specificity?

The *L6-L2H/Bgl* allele clearly retains *L6* gene-for-gene specificity. In those transgenic lineages where the autoactive phenotype is not expressed and in transgene heterozygotes of the JE3170 lineage that have a wild-type stature, transgenic plants are resistant to *L6*-avirulent rusts and susceptible to *L6*-viru-

lent rusts. For *L6MAD* plants, the conclusion is less clear. Because the reactions of *L6* and *L6MAD* plants to avirulent rusts is identical (minute HR flecks and no rust sporulation) and the reactions of *L6MAD* plants to *L6*-avirulent and *L6*-virulent rust strains can be distinguished on younger leaves on the basis of the amount of rust uredospore formation and chlorosis, we favor the view that the *L6MAD* protein coexpresses *L6* gene-for-gene specificity together with autoactivity. However, our inability to demonstrate clear reaction of these plants to the *Agrobacterium*-delivered *AvrL567-a* avirulence gene, possibly due to resistance to *Agrobacterium*, leaves some uncertainty about this allele's ability to recognize the avirulence gene product.

The dwarf phenotype.

One intriguing feature of flax plants transgenic for autoactive *L6* alleles is the dwarf phenotype. In its mild form, e.g., plants expressing *L6-L2 H/Bgl* and *L6MAD*, plants have a reduced internode length but have an otherwise normal phenotype and are fully fertile. The dwarf phenotype is not counteracted by application of the gibberellin GA3 (J. Ellis, unpublished results). The phenotypes of homozygous *L6MHV* flax and *Arabidopsis* lines homozygous for *ssi4* (Shirano et al. 2002), *adr1* (Grant et al. 2003), *bal* (Stokes et al. 2002), *ssi1* (Shah et al. 1999), and *snc1* (Zhang et al. 2003) are more extreme and, in *Arabidopsis*, include curled leaves. These differences probably result from an increased flux through the signal pathway downstream of the active R protein. The dwarf phenotype in several of the *Arabidopsis* mutants referred to above is associated with increased levels of salicylic acid (SA) and can be mimicked in transgenic plants expressing a bacterial gene for SA synthesis (Mauch et al. 2001). The dwarfing effects may be due to the "metabolic load" of constitutively expressing defense proteins such as PR1 (Heil and Baldwin 2002). The results of Tao and associates (2000) indicate another possibility. They showed that overexpression of *Arabidopsis* resistance gene *RPS2* in transient assays gives rise to an "overdose" effect that is correlated with decreased generalized de novo protein synthesis. This is reminiscent of plant stress responses, such as anaerobic stress that both induces the transcription of anaerobic response genes and stops translation of "nonanaerobic" messenger RNA (Sachs et al. 1980). A similar translational response may occur during defense activation. The dwarf phenotype in plants with autoactive R proteins may be derived from both metabolic load of defense protein expression and a general, partial reduction of normal nonstress protein synthesis through a translational control.

Auto-active *L6* confers resistance to rust strains virulent to *L6*.

Most autoactive variants of other NBS-LRR R proteins cause increased resistance to normally virulent pathogens (Grant et al. 2002; Li et al. 2001; Shirano et al. 2002; Stokes et al. 2002; Zhang et al. 2003), but we find no reports of autoactive resistance being associated with HR induced by the pathogen at the infection site. Indeed, Li and associates (2001) report the contrary for the interaction of *Arabidopsis* and *Peronospora parasitica* in *snc1* mutants. However, there has been one report of resistance in *Arabidopsis* to an otherwise virulent *P. parasitica*, induced by the chemical BTH, being associated with necrosis of host cells containing haustoria (Lawton et al. 1996). BTH induces defense gene expression and systemic acquired resistance. Resistance in autoactive flax plants to *L6*-virulent rust strains was associated with clear HR. Perhaps even in the absence of effective resistance genes, HR may be a "default" reaction of flax to infec-

tion by flax rust. In compatible interactions "default" HR may be actively inhibited by pathogen-derived virulence factors that block host defenses at the infection site. Under this scenario, chemically induced or autoactive host defense responses may slow pathogen metabolism and growth sufficiently to counter the pathogen's ability to subvert basal resistance and prevent the default HR. Alternatively, rust infection may induce a localized increase in expression of the mutant *L6* allele, the signal transduction components at the infection site that raises the level of these proteins over the threshold for cell death, or both. Autoactive resistance proteins may therefore have application in broad pathogen protection of transgenic crops if negative features such as the dwarf phenotype can be controlled.

MATERIALS AND METHODS

Plant material and rust strains.

Flax lines and flax rust strains have been described previously (Ellis et al. 1999; Lawrence et al. 1981). Transformation procedures for the flax line Ward have been described (Anderson et al. 1997). *Arabidopsis* was transformed by the silwet-mediated floral dip method (Clough and Bent 1998).

Agrobacterium-mediated transient expression assays.

Binary vectors were transformed into *Agrobacterium tumefaciens* GV3101pMP90 by triparental mating. Strain C58C1 was used as a Ti plasmid-free strain to demonstrate the requirement of T-DNA transfer for the necrotic phenotype. The presence of the correct vectors in strains was checked by isolating DNA from the *Agrobacterium*, electroporating it into *Escherichia coli*, and isolating and sequencing DNA from the latter bacterium. Strains were prepared for transient assays by growth overnight (28°C and 200 rpm) in 50-ml cultures of Luria broth containing gentamycin (0.05mg/ml), tetracycline (0.0035mg/ml), and 3 mM acetosyringone (Aldrich, St. Quentin, France). Cultures were centrifuged in a SS34 rotor (20°C and 8,000 rpm) for 10 min, and then were resuspended in Murashige-Skoog medium at an optical density at 600 nm of approximately 1.0. After incubation at room temperature for 1 h, they were infiltrated by gentle pressure injection with a needleless syringe into the underside of tobacco leaves or cotyledons of flax seedlings. *N. tabacum* cv. Samsun NN plants were generally 4 to 6 weeks old, *N. benthamiana* plants were 6 to 8 weeks old, while the flax plants were 14-day-old seedlings. Usually, 2 to 3 weeks was necessary to observe a strong response in the tobacco species, while necrosis of the flax cotyledons was observable within 10 days.

General nucleic acid techniques.

All sequencing was performed on an Applied Biosystems (Foster City, CA, U.S.A.) 377 sequencer, and individual sequences were aligned and assembled by the use of the Sequencher program (Gene Codes Corporation, Ann Arbor, MI, U.S.A.). Oligonucleotide site-directed mutagenesis was conducted by the Gene-editor kit (Promega, Madison, WI, U.S.A.) or by the method of Kunkel and associates (1991). Primers used for mutagenesis in this study are as follows:

P1 GATGAGTTTAAAAAGCACGACCAACTTAGAGAT
P2 GATGAGTTTAAAAATGGCCGACCAACTTAGAGAT
P3 GAGTTTAAAAATGCACGTCCAACCTTAGAGATATG
P4 GAGTTTAAAAATGCACAACCAACTTAGAGATATG
P5 GAGTTTAAAAATGCACCGCCAACCTTAGAGATATG
P6 GAGTTTAAAAATGCACTCCCAACTTAGAGATATG
P7 GAGTTTAAAAATGCACCTCCAACCTTAGAGATATG
P8 TGTTTCTTGAATTAGTCAGATCTGAGATACCTCCATG
P9 GTGGAATAGGCATGACGACCACTGC

Construction of binary vectors carrying L6 MHD mutants.

The *EcoRI-SphI* fragment of the *L6* gene (Lawrence et al. 1995) cloned in Bluescript (Stratagene, San Diego, CA, U.S.A.) was used for generating mutants of the *L6* MHD motif. Primers P1 to P7 were used to create the mutations M539K, H540A, D541V, D541N, D541R, D541S, and D541L, respectively. Clones containing the mutations were confirmed by DNA sequencing, cleaved with *EcoRI* and *SphI*, and ligated into full-length *L6* genomic sequence and were similarly digested. The full-length clone extended from a *SacI* site 5' of the ATG codon to a *BglIII* site 3' of the stop codon and was cloned as a *SacI-BglIII/BamHI* fusion clone in Bluescript. These mutated clones were then cleaved with *SacI* and *SmaI* and were ligated into either the binary vector pTNTRegL6ΔEB (endogenous *L6* promoter) or pTNNot35SL6 (35S CaMV promoter). Both binary vectors were prepared for cloning by digestion, first with *BglIII*, filling-in with dNTPs, and then by digestion with *SacI*. Finally, for the transformation of flax and *Arabidopsis*, a unique *PstI* site in both vectors was used for the cloning of the 35S CaMV-driven spectinomycin and bialaphos (*bar*) resistance genes, respectively.

L6 with mutant P-loop and with no LRR.

The *EcoRI-SphI* fragment of *L6* and the *L6* D541V mutation were used to generate the constructs coding for the *L6* protein devoid of the LRR region. In both clones, the codon for amino acid 604 was converted to an in-frame stop codon followed almost directly by a *BglIII* site, by the use of the primer P8. The fragments were sequenced and ligated into the *L6* genomic backbone, then were digested with *SacI* and *BglIII* and were ligated into the vector pTNTRegL6ΔEB, cleaved with the same enzymes. Substitution of the critical lysine residue in the P-loop sequence with methionine (K271M) was created by mutagenesis of Bluescript clones (*SacI-BglIII/BamHI* fusion) of the entire wild-type *L6* and *D541* mutant. These were sequenced, were cleaved with *SacI* and *SmaI*, and were ligated into pTNTRegL6ΔEB, which was prepared for cloning by digestion with *BglIII*, filling-in, and then, digestion with *SacI*.

Construction of L6-L2 H/Bg and L6-L10 H/Bg alleles.

The 3' 175-bp region of *L6* cloned into a binary vector (Ellis et al. 1999) was excised using a unique *HindIII* site in the coding region and a unique *BglIII* site downstream of the stop codon and was replaced with the corresponding fragments from *L2* and *L10*.

Construction of binary vectors coding for ethanol-inducible L6, L6MHV, and Fis1L6MHV.

SacI-BglIII/BamHI fusion clones of the entire genomic wild-type and that containing the D541V mutant *L6* genes were digested with *SacI* and *SmaI*, were filled-in with the Klenow fragment and dNTPs, and were ligated to the vector pACN, which had been digested with *SalI* and filled-in in a similar manner. Each of the resulting clones was partially digested with *HindIII*, and the fragments containing the *L6* sequences and flanking *alc* promoters and *nos* terminators were ligated into the binary vector pbinSRNACatN, which was prepared for cloning by digestion with *HindIII*. Plasmids pACN and pbinSRNACatN (Caddick et al. 1998) were obtained from and used under agreement with Syngenta Jealott's Hill (Berkshire, U.K.). The *Fis1L6MHV* vector was constructed as described previously (Ayliffe et al. 2002).

RNA extraction and Northern analysis.

Total RNA was extracted by a guanidinium isothiocyanate method (Chomczynski and Sacchi 1987). Samples were elec-

trophoresed on a 1% formaldehyde gel and were transferred to Hybond-N (Amersham, Tokyo) by standard procedures (Sambrook and Russell 2001).

PR1 expression in flax.

A flax PR1 homologue probe for gene expression analysis was amplified by PCR from genomic DNA of the flax line Ward. The redundant primers, containing inosine, were based on conserved regions in PR1 proteins from several plant species and the sequences were GCICA(A/G)(A/G)A(T/C) (T/A) (C/G)IC (A/C) ICA(A/G)GA(T/C)TA and CCAIACIA(C/T) (C/T) TGIGT (A/G)TA(A/G)TG. PCR conditions were 94°C for 2 min and then 40 cycles of 94°C (20 s), 46°C (20 s), 72°C (55 s). The reaction products were cloned into the vector pGEMT (Promega), and clones were confirmed by sequencing. Two approximately 320-bp products were isolated, and both encoded related PR1 homologues, as determined by Blast P analysis after removing the amino acid sequences encoded by the primer sequences. Clone 13 (NCBI accession number AY559144) was used in RNA gel blot analysis. PR1 expression in flax was induced using BTH (1,2,3-benzothiadiazole-7-carbothioic-acid-S-methyl ester) as Bion 500 WG, supplied by Novartis Crop Protection Australasia Pty Ltd. (Pendle Hill, Australia).

Chitinase assay.

The chitinase assay is described by Wirth and Wolf (1990). Leaf tissue (approximately 0.2 g of fresh weight), comprising the young, developing leaves at the tip of the plant and eight to ten fully expanded leaves below the tip, was collected from flax plants. These leaf samples were ground in Eppendorf tubes with buffer (200 µl, 0.05 M sodium acetate, pH 5.0) and a small quantity (approximately 20 mg) of a 1:1 mixture of acid-washed sand and insoluble polyvinyl pyrrolidone. More buffer (800 µl) was added, and the mixture was centrifuged and kept on ice until required. Chitinase activity was determined using carboxymethylchitin-remazol brilliant violet solution (2 mg ml⁻¹) (Loewe Biochemica, Munich) as substrate. After incubation (30 min at 37°C), undigested substrate was precipitated with HCl and was removed by centrifugation. An estimate of the chitinase activity was made by measuring the difference between the absorbance at 540 nm (A_{540}) values of an aliquot of supernatant from a digested and an undigested reaction (when HCl was added immediately). Protein concentrations in the diluted extracts were determined, using a bicinchoninic acid kit (Pierce, Rockford, IL, U.S.A.) according to the manufacturer's directions. Chitinase activities were expressed as ΔA_{540} obtained per milligram of soluble protein.

Ethanol induction of *alcA*-driven constructs in transgenic *Arabidopsis* and flax.

Transgenic plants containing the *alcA/alcR* gene expression systems were grown in soil under greenhouse conditions of 22°C day and 18°C night. A 12-h-day period was used for *Arabidopsis*. Approximately 4- to 5-week-old plants were sprayed with a 2% ethanol spray (Roslan et al. 2001), were covered with plastic for 24 h, and then, their response to the treatment was observed. Ethanol induction was also done with transgenic plants growing in sterile tissue culture. *PR1* and *L6* gene expression in these plants was detected, using the coding regions of the genes amplified from genomic DNA of *Arabidopsis* and the cloned coding region of *L6*, respectively.

Autofluorescence assay.

Five days after treatment (rust and mock infections), flax leaves were removed from the plant, were boiled 2 to 3 min in absolute ethanol with a few glass beads. The leaves were then

placed in a saturated aqueous solution of chloral hydrate (Sigma, St. Louis) at room temperature. Cleared leaves were viewed under a microscope with a UV light source.

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