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Rapid activation of a novel plant defense gene is strictly dependent on the *Arabidopsis RPM1* disease resistance locus

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We cloned and sequenced cDNAs encoded by a novel plant defense gene, *ELI3*, from parsley and *Arabidopsis thaliana*. The predicted product shares no homology to known sequences. *ELI3* mRNA accumulates in *A.thaliana* leaves in response to challenge with phytopathogenic *Pseudomonas syringae* strains. The timing and magnitude of this response are dictated by the genetics of the plant-pathogen interaction being analyzed. During incompatible interactions, where resistance in the plant genotype Col-0 is dictated by the dominant *RPM1* locus, *ELI3* mRNA accumulates to high levels 5-10 h post-inoculation. This kinetic behavior is also generated by the presence of a cloned bacterial avirulence gene, in otherwise virulent bacteria, which triggers resistance mediated via *RPM1* action. The phenotypic outcome is a hypersensitive resistance reaction visible 8-15 h post-infiltration. Thus, the induction kinetics of *ELI3* mRNA accumulation are consistent with a functional role for the *ELI3* gene product in establishing the resistant phenotype. In contrast, during compatible interactions with the susceptible plant genotype Nd-0, which is homozygous recessive at the *rpm1* locus, *ELI3* mRNA accumulates significantly only after 15 h. We show genetically that *ELI3* activation is strictly dependent on the presence of dominant alleles at *RPM1* using an assay generalizable to any pathogen induced plant defense phenomena.

Key words: Arabidopsis/bacterial avirulence gene/disease resistance gene/plant defense/*Pseudomonas syringae*

Introduction

Plants have evolved a large array of active defense mechanisms, many of which are accompanied by *de novo* transcription of an equally large number of 'plant defense genes'. The functions encoded by these genes are diverse. They range from enzymes involved in the biosynthesis of potentially protective antimicrobial phytoalexins, through proteins putatively deployed *in situ* as antimicrobial degradative enzymes, to structural proteins used in strengthening neighboring undamaged tissue against the onslaught of the pathogenic aggressor. Nearly all plant defense genes also have developmentally regulated cell-type specific functions during plant growth. In all pathosystems analyzed by molecular methods to date, many genes whose transcriptional activity is induced upon pathogen ingress have been identified and characterized. In no case, however, has an obvious role in establishment or maintenance of the resistant phenotype been ascribed to an activated plant defense gene on the basis of its known biochemical function. Nor has an induced gene activity ever been shown to be causally necessary for a resistance reaction (Crute *et al.*, 1985; Collinge and Slusarenko, 1987; Hahlbrock and Scheel, 1989; Lamb *et al.*, 1989; Dixon and Harrison, 1990; Dixon and Lamb, 1990; Dangl, 1992a).

Genetic control of plant disease resistance is also well documented. Experiments in crop species for over 50 years clearly demonstrate that resistance to many important pathogens is controlled by single, dominant loci in the plant (*R*-genes), each of whose product interacts, either directly or indirectly, with the product of a dominant avirulence (*avr*) gene in the pathogen (Crute, 1985; Ellingboe, 1981, 1982, 1984; Flor, 1955, 1971; Keen, 1982, 1990; Keen and Staskawicz, 1988; Knogge, 1991). Analysis of activation of plant defense gene transcription in the context of genetically defined interactions has often been undertaken. In one typical experimental scenario, plant defense gene mRNA accumulation is measured after inoculation of a single pathogen isolate onto either resistant or susceptible plant cultivars. An alternative experimental regime employs two non-isogenic pathogen isolates, one of which is virulent and the other avirulent, on a single plant cultivar. In many cases, using either type of experiment, mRNA accumulation for a given plant defense gene is very rapid (and often transient) during a resistance reaction, but markedly slower in susceptible reactions (e.g. Bell *et al.*, 1984, 1986; Fritzemeier *et al.*, 1987; Habereder *et al.*, 1989; Voisey and Slusarenko, 1989). Differences in magnitude between levels of accumulation in resistant and susceptible plant genotypes are also often observed, but not universally. Most interpretations of results from these two experimental regimes argue that the presence of an *R*-gene determines the rapidity of the spectrum of plant defense responses, and that this differential timing is the critical variable in the outcome of the interactions. Thus, a plant defense gene whose product plausibly plays a causal role in the resistance reaction triggered through specific *R*-gene function should be exclusively or at least preferentially activated during the resistance reaction, and its activation should precede the phenotypic manifestation of resistance.

These sorts of analyses are severely limited, however, by genetic heterogeneity of either plant cultivars or pathogen isolates, or both. In particular, if resistant and susceptible plant cultivars are inoculated by a single pathogen isolate and a given induced defense response assayed, there is no control for the effect of plant genetic background on the outcome. The use of near-isogenic plant genotypes, ostensibly differing at only one *R*-gene-containing locus, minimizes this problem (Davidson *et al.*, 1987, 1988) but does not remove it altogether, since genetic drag of unlinked

loci during backcrossing is well documented (Young and Tanksley, 1989). Also, use of genetically diverse pathogen genotypes, which often have distinct growth and differentiation time course in their host, can confound interpretation of induced defense activities (e.g. Mahé *et al.*, 1992). We have taken an alternative approach to show here that rapid activation of a novel plant defense gene, *ELI3*, is strictly dependent on the presence of a specific plant *R*-gene, and that its induction is concurrent with onset of the resistant phenotype. These findings support a functional role for the ELI3 gene product in disease resistance. This assay is simple and generally useful for establishing 'functional co-segregation' of activated plant defense responses and *R*-gene action. We have used it to test *R*-gene mediated activation of many other plant defense genes, of both known and unknown biochemical functions.

Results

Isolation and characterization of ELI3 cDNA clones

We used the previously described parsley *ELI3* cDNA (Somssich *et al.*, 1989) as a heterologous probe on a genomic Southern blot of *A.thaliana* DNA to determine whether cross-hybridizing sequences were present. Figure 1A shows the result, which suggests that the complexity of *ELI3* hybridizing signals in *A.thaliana* under moderate stringency is low. A 3.8 kb *Hin*dIII fragment was subsequently cloned from an *A.thaliana* genomic library (Trezzini *et al.*, 1992) and we used it to probe a homologous Southern blot (Figure 1B). It detects a single fragment in genomic DNA from each of three *A.thaliana* genotypes, after digestion with any of several restriction enzymes. This clone, and the parsley cDNA clone, were we used it to screen an *A.thaliana* cDNA library. Two different full-length *A.thaliana* cDNAs were characterized. They are tightly linked in the genome, separated by only ~2.0 kb (S.Kiedrowski and J.L.Dangl, unpublished). Preliminary RFLP mapping data place the *ELI3* genes on *A.thaliana* chromosome 4 (T.Debener, unpublished). Their cDNA sequences, and the sequence of the single parsley cDNA analyzed to date, are shown in Figure 2A. Deduced amino acid sequences for all three clones are compared in Figure 2B. The presence of one long open reading frame, beginning with a nearly perfect translational context (Kozak, 1984; Joshi, 1987), orients the cDNA sequences at the 5′ end; poly(dA) tails define the 3′ end.

Although we have not determined the transcriptional start point, the sequence at positions 1−6 of both cDNAs (and surrounding these positions in the genomic clones, S.Kiedrowski, unpublished) is highly similar to others known from plant genes (Breathnach and Chambon, 1981); Joshi, 1987). The putative 5′ untranslated leader is extremely A-T rich (75%), another typical feature of plants (Joshi, 1987). The predicted apparent molecular weights of 44.5 kDa and 45 kDa for the two *A.thaliana* isoforms is in good agreement with *in vitro* translation data from parsley cultured cells (43 kDa; Somssich *et al.*, 1989). As shown below, the cDNA size of 1.3 kb is also consistent with measurements of the mRNA size by Northern blot analysis. The two *A.thaliana* clones are 84% identical at the nucleotide level, and 85% identical (93% similar) at the amino acid level. They are each 67% identical to the parsley cDNA sequence, and share 70% amino acid identity (83% similarity). No match or significant partial homology was found when these sequences were searched against the EMBL, SwissProt and PIR databases.

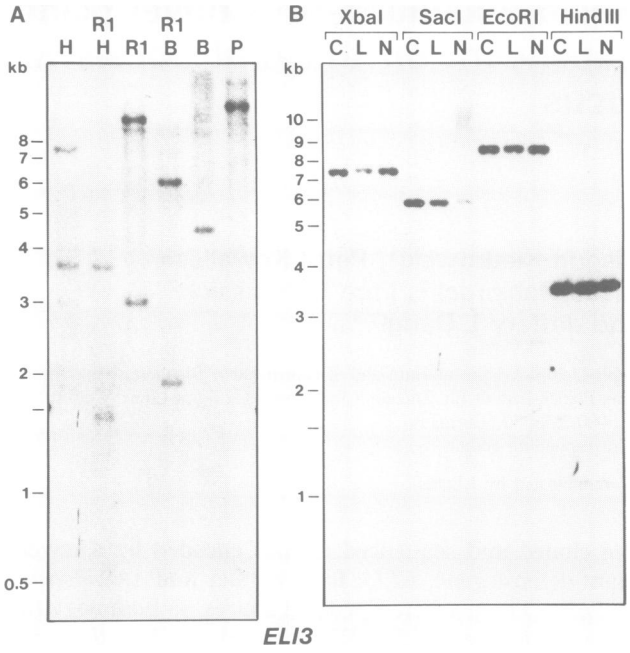

Fig. 1. Genomic complexity of *A.thaliana ELI3* genes. (**A**) Genomic Col-0 DNA digested with various restriction enzymes, probed with the near full-length parsley *ELI3* cDNA using moderate stringency conditions (see Materials and methods). (**B**) The 3.8 kb *Hin*dIII fragment hybridizing in panel A was cloned and used as probe on Southern blots of three *A.thaliana* genotypes, under high stringency conditions (see Materials and methods). H, *Hin*dIII; R1, *Eco*RI; B, *Bam*HI; P, *Pst*I; C, Col-0; L, La-er; N, Nd-0

Activation of ELI3 by phytopathogenic bacteria

We have previously shown that *ELI3* mRNA accumulates after infiltration of *A.thaliana* leaves with a variety of phytopathogenic bacteria (Dangl *et al.*, 1991). These preliminary studies also showed that the amount of *ELI3* mRNA in uninoculated or mock inoculated leaves was very low. With this background information, we pursued the control of *ELI3* activation in detail using the genetically defined pathosystem outlined in Figure 3. The *A.thaliana* ecotype Col-0 is resistant to the *Pseudomonas syringae* pv. *maculicola* (Psm) isolate m2. The interaction between them is incompatible, and the outcome is a typical hypersensitive resistance reaction (HR). In contrast, the *A.thaliana* genotype Nd-0 is susceptible to Psm m2, the interaction is compatible, and the bacteria grow to high density *in planta* and cause disease symptoms. Both plant genotypes are susceptible to bacterial isolate Psm m4. These combinations of plant and bacterial genotypes were previously used to identify the single plant resistance gene, *RPM1*, and the corresponding bacterial avirulence gene, *avrRpm1* (Debener *et al.*, 1991). The presence of *avrRpm1* in Psm m4 on a broad host range cosmid is sufficient to render it avirulent on Col-0, where its function is recognized by the *RPM1* function.

The ability of these three bacterial strains to activate *ELI3* expression was measured over a 24 h time course after infiltration into either Col-0 or Nd-0 leaves. This time course was chosen since the HR observed on Col-0, under these assay conditions, is macroscopically visible between 10 and 15 h. Figure 4 shows RNA blot analysis of *ELI3* mRNA accumulation with the three bacterial strains described in

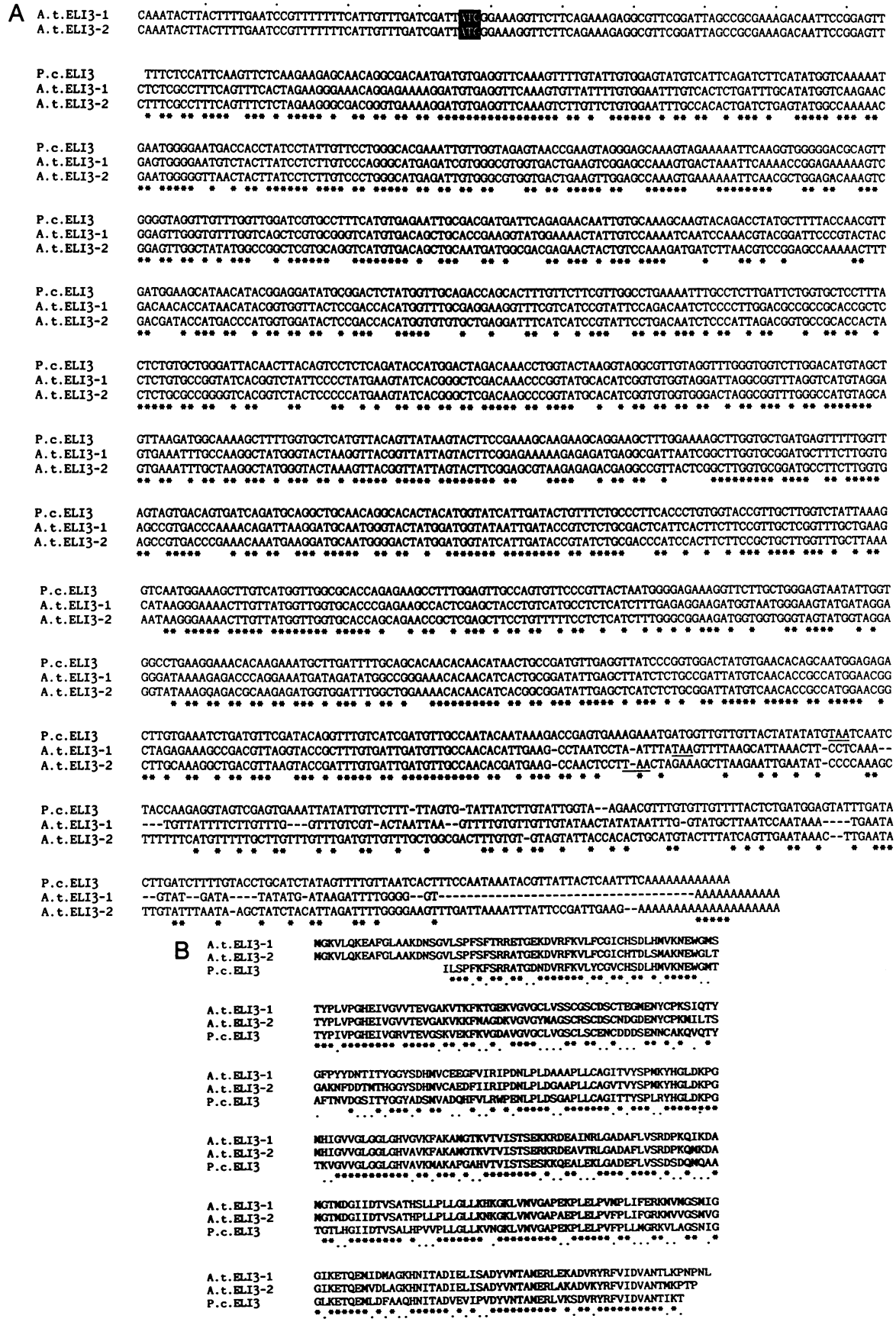

Fig. 2. (**A**). Nucleotide sequence comparison of *A.thaliana* (A.t.) and parsley (P.c.) *ELI3* cDNAs. The two *A.thaliana* sequences begin 106 nt 5′ of the incomplete parsley sequence. The putative translation initiation codons are blocked in black, and the first in-frame translation stop for each cDNA is underlined. Asterisks indicate conservation among all three sequences; dashes are gaps introduced for best alignment. (**B**) Deduced amino acid three sequences; dots represent conservative amino acid exchanges. The sequences have been deposited in the EMBL and GenBank databases and are available under accession numbers X67815 (*ELI3-2*), X67816 (*ELI3-1*) and X67817 (P.c. *ELI3*).

Arabidopsis genotype:	Col-0	Nd-0
P. s. maculicola:		
m2	I	C
m4	C	C
m4/ *avrRpm1*(m2)	I	C

Fig. 3. Summary of plant−pathogen interactions discussed in this paper. The outcome of interactions between plant genotypes and pathogen isolates is either incompatible (I; plant resistant, pathogen avirulent, HR$^+$) or compatible (C; plant susceptible, pathogen virulent, HR$^-$).

Figure 3. After challenge with Psm m2, or with a Psm m4 transconjugant harboring the *avrRpm1* gene derived from Psm m2, *ELI3* mRNA accumulates to high levels by 5 hours post-inoculation (h.p.i). In contrast, similar levels are reached only between 15 and 24 h.p.i. after inoculation with the virulent Psm m4 strain. Quantification of these data by densitometric scanning shows a 25-fold preferential induction in the incompatible interaction at 10 h.p.i. At 5 h.p.i. this value is at least 50-fold. A similar experiment for the Nd-0 plant genotype is shown in Figure 4. Inoculation with any of the three test bacterial strains results in a compatible interaction, since Nd-0 is susceptible and homozygous recessive at the *rpm1* locus. Very little *ELI3* mRNA accumulates during the interaction with Psm m2, in contrast to the large amount induced by either Psm m4, or the Psm m4 transconjugant carrying *avrRpm1*. The presence of the *avr* gene appears to slow the kinetics of *ELI3* mRNA accumulation slightly. In no case, however, is there a response as rapid as those in which Col-0 is resistant to the challenge bacteria (Figure 3).

Debener *et al.* (1991) identified and mapped the *RPM1* locus using a segregating population derived from a Col-0×Nd-0 cross, through screening for generation of HR after challenge with Psm m2. *RPM1* segregated as a dominant, single locus dictating formation of an HR against Psm m2 in these experiments. Data presented in Figure 4 show that the difference in *ELI3* mRNA accumulation between Col-0 and Nd-0, with respect to both timing and magnitude, is very large (at least 20-fold at 10 h.p.i.) after inoculation with Psm m2. We therefore devised an experiment to ask whether the early, high level *ELI3* activation in Col-0 after Psm m2 challenge is dependent on dominant *RPM1* function. F2 individuals from the Col-0×Nd-0 cross were previously allowed to self-pollinate, and F3 families were screened to establish the F2 genotype at *RPM1* (Debener *et al.*, 1991). Families had been identified derived from uniformly resistant (*RPM1/RPM1*) or uniformly susceptible (*rpm1/rpm1*) F2 homozygotes, or segregating heterozygote F2 plants. We reasoned that if rapid, high level *ELI3* induction is dependent on dominant *RPM1* function, then all *RPM1/RPM1* families would show the mRNA accumulation kinetics observed in the resistant Col-0 parent. Conversely, all *rpm1/rpm1* families would generate the kinetic of the susceptible Nd-0 parent.

Nine individuals from each of 15 homozygous resistant

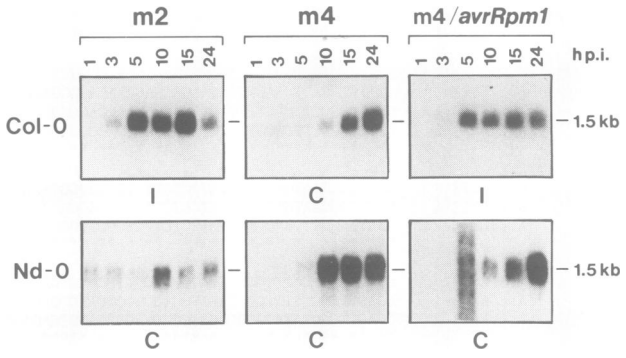

Fig. 4. Pathogen-induced accumulation of *A.thaliana ELI3* mRNA is dependent on the genetics of the plant−microbe interaction. Leaves of either Col-0 or Nd-0 plants were infiltrated with one of three *P.syringae* pv. *maculicola* bacterial strains (see Figure 3), and harvested at the indicated time point (hours post-inoculation). RNA blots were probed with cDNA insert from the *A.thaliana ELI3-1* gene. I, incompatible interaction; C, compatible interaction (see Figure 3). The RNA sample in the 5 h.p.i. lane of the Nd-0 m4/*avrRpm1* interaction is degraded.

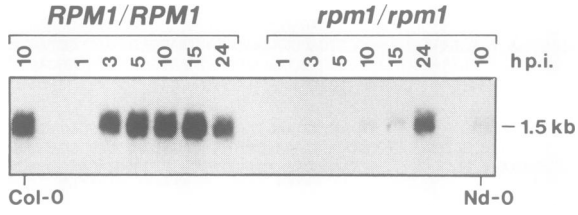

Fig. 5. *ELI3* induction in two (Col-0×Nd-0) F3 families. Leaves from nine individuals from a single, randomly chosen family of each homozygous class, and either Col-0 or Nd-0 parental plants, were infiltrated with Psm m2 (see Materials and methods). Time course of harvest and blot analysis were as described for Figure 4.

and 15 homozygous susceptible familes were inoculated with Psm m2. Pooled leaves from all nine individuals within each family were used for RNA preparation. A complete set of kinetic data for one family of each class is shown in Figure 5. Early, high level *ELI3* mRNA accumulation is apparent in the *RPM1/RPM1* family and not in the *rpm1/rpm1* family. This experiment shows, importantly, that although the two families were genotype-selected only at the resistance gene locus and are freely assorting at all other loci, the *ELI3* induction kinetics mirror those of the respective parental plant genotypes. We chose 10 h.p.i. as the time point for analysis of the remaining families, since the level of *ELI3* in *rpm1/rpm1* families is obviously measurable, but still much lower than in *RPM1/RPM1* families. RNA blot results are shown for seven families of each homozygous class in Figure 6, and densitometric quantification of all 30 families is graphically illustrated in Figure 7. These data clearly show that the early, high level accumulation of *ELI3* mRNA functionally co-segregates with *RPM1*. The quantitative differences between homozygous resistant and homozygous susceptible families are obvious in the autoradiogram, where uniform levels of *ELI3* mRNA, either high or low, are apparent. The mRNA levels in each family also reflect those present in the respective parent plant genotypes, as evidenced by the control lanes in Figures 4−6. Also, standard deviations from mean *ELI3* mRNA levels for *RPM1/RPM1* or *rpm1/rpm1* families are clearly non-overlapping.

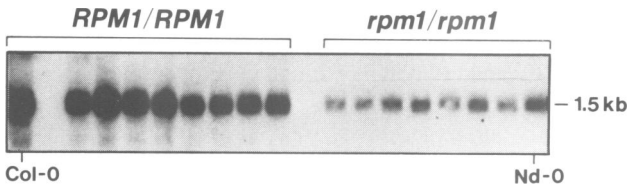

Fig. 6. Early *ELI3* induction cosegregates with *RPM1* function. Leaves of nine individuals from seven families of each homozygous class, and either Col-0 or Nd-0 parental plants, were inoculated with Psm m2 (see Materials and methods). All leaves were harvested 10 hours post-inoculation. RNA blots were prepared and hybridized as described for Figure 4.

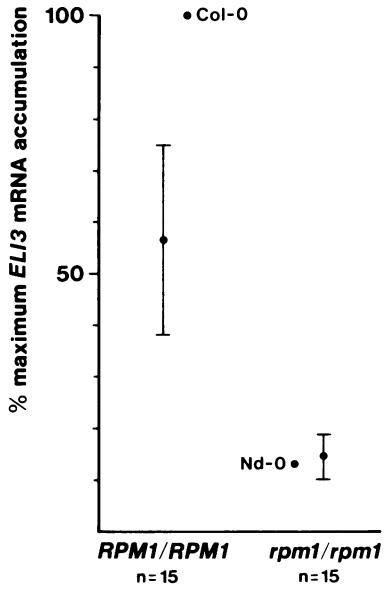

Fig. 7. Quantitative analysis of early *ELI3* induction from 30 (Col-0×Nd-0) F3 families. Hybridization signals as presented in Figure 6 were quantified by densitometric scanning of the autoradiographic films. Each of the 30 F3 families was analyzed on two independent RNA blots and average values were calculated. These averages are plotted as mean (dot) ± standard deviation relative to the Col-0 parental level. Parental values are the mean of four independent measurements. The observed absolute ranges for Col-0 were ± 4.7%; those for Nd-0 were ± 2.6%.

We have also tested an available panel of 15 other plant defense genes (see Trezzini *et al.*, 1992 for listing) to address whether they meet our first criterion for possible causal involvement in the maintanence and establishment of resistance in this pathosystem, namely exclusive or preferential activation during incompatible interactions. Other than *ELI3* mRNA, only mRNAs for phenylalanine ammonia-lyase (PAL) and tyrosine decarboxylase (TDC) exhibit preferential, early accumulation during the incompatible interaction detailed above (data not shown). For all other cases tested, either the genes were constitutively expressed in *A.thaliana* leaves, or no mRNA was detected at any time point after bacterial inoculation. Preliminary evidence suggests that tyrosine decarboxylase mRNA accumulation is dependent on *RPM1*. *PAL* mRNA accumulation levels during the incompatible interaction are only 3- to 5-fold above those observed during the compatible interaction, thus greatly complicating the functional co-segregation test.

Discussion

Our data are the first to indicate clearly that there is a functional linkage between an *R*-gene mediated trigger of plant disease resistance and the activated defense responses thought to be causal in the establishment of the resistant phenotype. Although not entirely unexpected in light of the weight of available data, these results prove that *R*-gene-dependent cognitive function results in plant defense gene activation. The simple experimental outline we followed is generalizable to many aspects of active plant defense mechanisms.

Three conclusions can be drawn regarding the pathogen induced expression of the *ELI3* gene from the data presented in Figure 4. First, *ELI3* mRNA accumulates very rapidly to high levels in the incompatible interaction between Psm m2 and Col-0, in a time frame preceding development of macroscopic HR. Secondly, the *avrRpm1* gene, derived from Psm m2, is sufficient to trigger this early induction when present in an otherwise virulent background. Thirdly, compatible interactions with non-isogenic bacteria can have different effects on *ELI3* activation during the early course of disease onset. Our most important conclusion, that early *ELI3* activation is strictly regulated by the *RPM1* disease resistance gene, is supported by functional co-segregation data summarized in Figure 7. Importantly, these data are statistically very robust [$P = 10^{-9}$; calculated by $P = 2[1 - (1/4^n)(1/4^n)]$ where n = number of families in each homozygous class; Allard, 1956; Michelmore *et al.*, 1991]. Apparent differences between *ELI3* mRNA levels in *RPM1/RPM1* families and the Col-0 parent could be indicative of other factors influencing overall expression. These factors are presumably segregating freely in the pools of F3 individuals that made up each RNA sample.

A plethora of studies have shown that plant defense genes are often preferentially expressed early during incompatible interactions (Crute *et al.*, 1985; Collinge and Slusarenko, 1987; Hahlbrock and Scheel, 1989; Lamb *et al*, 1989; Dixon and Harrison, 1990; Dangl, 1992a,b). In a typical example, Bell *et al.* (1984, 1986) analyzed the response of one bean cultivar to inoculation with either of two isolates of a fungal pathogen, one virulent and one avirulent. They showed that chalcone synthase (*CHS*) mRNA activity, and mRNA amounts for both *CHS* and *PAL*, accumulated more rapidly in tissue directly adjacent to fungal penetration sites during a resistance response. Nearly 5-fold more *CHS* mRNA activity accumulated during disease onset than during the resistance response, but at a much later time point (Bell *et al.*, 1984). Symptomatic of analyses of this type, however, little consideration was given to the possible effects of fungal genetic background. Recent analysis in this same pathosystem, using near isogenic bean cultivars carrying an introgressed *R*-gene, show little, if any, differential accumulation of several plant defense genes between resistant and susceptible isolines (A.Mahé and M.Dron, personal communication) and also show that the time course of gene activation is clearly influenced by fungal genetic background (Mahé *et al.*, 1992). Other experiments suffering from similar limitations have been reported from a variety of pathosystems (Fritzemeier *et al.*, 1987; Habereder *et al.*, 1989; Voisey and Slusarenko, 1989).

The application of near-isogenic plant cultivars, differing in the ability to recognize a single fungal isolate, to analysis

of induced defense gene activity has been reported by Davidson *et al.* (1987, 1988). 2- to 5-fold more mRNA for several barley genes of unknown function accumulated in resistant than in susceptible isolines. These authors' observations were strengthened by the fact that any one of three barley *R*-genes backcrossed into different susceptible backgrounds could induce the activity of two of the defense genes. Although these data are strongly suggestive of a role for *R*-genes as triggers of activated defense responses, the documented phenomena of linkage drag despite repeated backcrossing (Young and Tanksley, 1989) must be kept in mind. In cases of linkage drag, a gene or genes unlinked to the *R*-gene could be critical in determining the differential activation of a given defense gene.

Similar experiments have been performed using phytopathogenic Pseudomonads (Daniels *et al.*, 1987). The accumulation of mRNA for several plant defense genes encoding unknown function was measured in different pea cultivars after inoculation with different races of *P.syringae* pv. *pisi*. Although low levels of differential accumulation were measured, this system is hampered by different genetic backgrounds of both host and pathogen. Recent analyses using *A.thaliana* as a model have also been performed (Ausubel *et al.*, 1991; Dangl *et al.*, 1991; Dangl, 1992b; Davis *et al.*, 1991, 1992). As mentioned above, our preliminary kinetics showed that *ELI3* activation was earlier during incompatible interactions than during compatible ones (Dangl *et al.*, 1991). Davis *et al.* (1991) detailed the expression of *PAL* mRNA after challenge with a broad range of phytopathogenic Pseudomonads. These data indicated that *PAL* mRNA accumulates preferentially during incompatible interactions, and supported observations for *PAL* expression from several systems (Hahlbrock and Scheel, 1989; Lamb *et al.*, 1989; our unpublished data). Analyses of defense gene activation within a single plant genotype can be simplified and strengthened by challenge with isogenic phytopathogenic bacterial strains differing only by the presence or absence of a cloned *avr* gene. Davis *et al.* (1992) have recently shown that the presence of the cloned *avrRpt2* gene (Dong *et al.*, 1991; Whalen *et al.*, 1991) in an otherwise virulent *P.syringae* pv. *maculicola* strain is sufficient to shift the appearance of *PAL* mRNA to an early time point characteristic of most incompatible interactions. This observation was made using one resistant *A.thaliana* genotype. Surprisingly, however, the same time shift of *PAL* mRNA accumulation was seen during a compatible interaction of the same *avrRpt2* containing strain with a second *A.thaliana* genotype. Our own data for *ELI3* (Figure 4, above) show clearly that different types of compatible interactions can generate different patterns of defense gene activation during the onset of disease. We propose that our non-isogenic Psm strains express different virulence activities in the compatible interactions we analyzed, giving rise to different levels of *ELI3* mRNA. We stress, however, that the activation of this, and other, plant defense genes during the onset of disease is probably independent of bacterially encoded *avr* functions mediating specific recognition by the plant.

The above discussion illustrates the advantages of genetically defined pathogens, assayed in combination with uniform genetic plant backgrounds. These considerations were critical in our choice of F3 families from F2 individuals which had been genotype-selected at the *RPM1* locus, but must be assumed to be freely segregating at all unlinked loci. This principle has also been applied in searching for DNA markers linked to known resistance genotypes within pools of plants from segregating populations (Michelmore *et al.*, 1991). In our experiments, pooled individuals from F3 families should have uniformly mixed resistant and susceptible genetic backgrounds, except where selected for a particular *RPM1* genotype. We also screened with the Psm m2 isolate which is known to contain only the *avrRpm1* gene capable of triggering resistance through *RPM1* (Debener *et al.*, 1991; C.Ritter and J.L.Dangl, unpublished), and which generates easily differentiable *ELI3* induction patterns.

The causal role of plant defense gene activation in the phenotypic outcome of a particular plant−pathogen interaction remains unanswered. This is true for genes encoding both known and unknown biochemical function. Genetic approaches, however, offer the most succinct dissection of the activated plant defense response thought to establish and maintain the resistant state. From data presented here, it is plausible that accumulation of the *ELI3* gene product is one, of potentially many, components necessary for the development of HR against *P.syringae* pv. *maculicola* strains capable of triggering *RPM1* function. This plausibility is born of the functional cosegregation of early, high level expression with *RPM1* function. To test critically the role of *RPM1*-dependent *ELI3* activation in disease resistance, we are currently creating *eli3* phenocopy mutants using antisense constructs. These will be analyzed for lack of induced *ELI3* mRNA levels and subsequent effect on the outcome of the plant−microbe interaction. If *ELI3* gene activation had not functionally co-segregated with *RPM1* activity, its potential importance in the specific resistance reaction would have evaporated.

Materials and methods

Genomic DNA isolation and analysis

Leaves of *A.thaliana* were harvested and genomic DNA was prepared using the CTAB method (Murray and Thompson, 1980). After restriction enzyme digestion of 3 μg aliquots, the samples were electrophoretically fractionated on 0.8% horizontal, native agarose gels and denatured as described by Sambrook *et al.* (1989); the DNA fragments were then transferred to Hybond-N nylon membranes (see 'RNA filter hybridization'). Prehybridization and hybridization were performed in 1 M NaCl, 10% dextran sulfate, 1% SDS, 100 μg/ml denatured salmon sperm DNA at 60°C. Washing was done at 60°C in 6×SSC, 0.5% SDS for 30 min and two washes in 1×SSC, 0.5% SDS for 30 min each for high stringency, or 60°C in 2×SSC, 0.5% SDS, twice for 30 min each for moderate stringency.

Library screening and cDNA characterization

A cDNA library in λgt10 of *A.thaliana* (accession Col-0) whole plants grown under sterile conditions, was kindly provided by Dr Andreas Bachmair, MPI Köln. 0.6×10^6 p.f.u. were screened with a 463 bp *Eco*RI−*Nco*I fragment from the 5′ end of the parsley *ELI3* cDNA as radiolabelled probe, under moderately stringent conditions (1 M NaCl, 10% dextran sulfate, 1% SDS at 60°C; washes, 30 min in 6×SSC, 0.5% SDS, and 2×30 min in 2×SSC, 0.5% SDS) Thirty positive phages were picked and purified. The identity of 14 of them was cross-confirmed via hybridization with a 1.1 kb *Xba*I−*Xho*I genomic *A.thaliana* genomic fragment containing a portion of the *ELI3* gene under stringent conditions. The insert size was determined by *Eco*RI digestion and the two largest inserts were cloned into Bluescript pKS+ and pUC19.

Cloning and sequencing

Standard cloning procedures (Sambrook *et al.*, 1989) and chain termination sequencing (Sanger *et al.*, 1977) using Sequenase version 2.0 (US Biochemical) were applied. In additional to the universal and reverse primers, new primers were generated complementary to the template sequence for subsequent sequencing steps using an ABI synthesizer.

Bacteria

Pseudomonas syringae pv. *maculicola* (Psm) strains were obtained from the LMG culture collection (Rijksuniversiteit Gent, Belgium). LMG 5071 (Psm m2) was isolated from *Brassica oleracea* var. Botrytris, and LMG 5295 (Psm m4) from *Raphanus sativus*. They were grown at 28°C in King's B (KB) medium (King *et al.* 1954) containing 100 μg/ml rifampicin. The transconjugant K 48/6 carries *avrRpm1* on pCR102 (gift of Claudia Ritter). This cosmid encodes resistance to 5 μg/ml tetracycline. *Escherichia coli* strains DH5 and MC 1061 were grown in Luria–Bertani (LB) medium (Sambrook *et al.*, 1989).

Plant maintenance, inoculation and leaf harvest

This was performed essentially as described by Dangl *et al.* (1991) and Debener *et al.* (1991). F3 and F4 families from the population segregating *RPM1* function from a (Col-0×Nol-0) cross were kindly provided by Thomas Debener (described in Debener *et al.*, 1991). For data shown in Figures 5–7, leaves from nine individuals from each family were pooled for RNA extraction. Plants were grown and treated as described elsewhere (Debener *et al.*, 1991) with the exception that half the leaf was inoculated with high density (10^8 c.f.u./ml in 10 mM $MgCl_2$) bacterial suspension. Infiltration with 10 mM $MgCl_2$ served as a wound control. After inoculation, the plants were covered and kept at nearly 100% humidity during the time period of analysis. The marked leaves were cut off the plants 1, 3, 5, 10, 15 and 24 h after infiltration, immediately wrapped in aluminium foil, frozen in liquid nitrogen and stored at −80°C.

RNA isolation

Frozen plant leaves were homogenized in liquid nitrogen using a mortar and pestle. The fine powder was added to precooled guanidinium buffer (8 M guanidinium hydrochloride, 20 mM MES; 20 mM EDTA pH 8.0; 50 mM β-mercaptoethanol). After addition of 1 vol of phenol–chloroform (1:1), the homogenate was shaken for 5 min and centrifuged for 7 min at 5000 *g*, at room temperature to separate the phases. The aqueous phase was extracted again with an equal volume of phenol–chloroform and, if no interphase was present, a last extraction with chloroform alone was performed. Nucleic acids were precipitated with 0.1 vol 3 M sodium acetate, pH 5.2 and 2 vol ethanol. After resuspension in 1 ml H_2O, RNA was selectively precipitated by addition of 1.5 ml 5 M $LiCl_2$, 20 mM sodium acetate, pH 5.2 and incubation on ice for >2 h. The RNA pellet was resuspended in 800 μl H_2O and reprecipitated with 0.1 vol 3 M sodium acetate, pH 5.2 and 0.7 vol isopropanol. Finally RNA was disolved in 200 μl H_2O and OD_{260mm} and OD_{280mm} were measured.

RNA filter hybridization

Total RNA (5 μg) was size fractionated on formaldehyde agarose gels (Lehrach *et al.* 1977) and blotted onto Hybond-N nylon membranes (Amersham Buchler, Braunschweig, Germany) with 10×SSC (1×SSC in 0.15 M NaCl, 0.015 M sodium citrate). After fixation (by UV irradiation using a Stratalinker, and baking at 80°C for 2 h), filters were stained with ethidium bromide and photographed to determine the amount of transferred RNA per lane. Prehybridization (2 h) and hybridization (16–18 h) were performed in 5×SSC, 5% dextran sulfate, 0.1% SDS, 100 μg/ml denatured herring sperm DNA at 65°C. Radiolabelled DNA probes were generated by the random oligolabelling technique (Feinberg and Vogelstein, 1983). Washes were in 1×SSC, 0.5% SDS for 30 min at 65°C. All filters were wrapped in Saran Wrap and exposed to Hyperfilm MP X-ray films (Amersham) at −70°C using intensifying screens (Trimax, 3M).

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