Constitutive gain-of-function mutants in a nucleotide binding site–leucine rich repeat protein encoded at the Rx locus of potato

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Summary

Rx in potato encodes a protein with a nucleotide binding site (NBS) and leucine-rich repeats (LRR) that confers resistance against Potato virus X. The NBS and LRR domains in Rx are present in many disease resistance proteins in plants and in regulators of apoptosis in animals. To investigate structure-function relationships of NBS-LRR proteins we exploited the potential of Rx to mediate a cell death response. With wild-type Rx cell death is elicited only in the presence of the viral coat protein. However, following random mutagenesis of Rx, we identified mutants in which cell death is activated in the absence of viral coat protein. Out of 2500 Rx clones tested there were seven constitutive gain-of-function mutants carrying eight independent mutations. The mutations encoded changes in the LRR or in conserved RNBS-D and MHD motifs of the NBS. Based on these findings we propose that there are inhibitory domains in the NBS and LRR. The constitutive gain-of-function phenotypes would be due to deletion or modification of these inhibitory domains. However activation of Rx is not simply release of negative regulation by the LRR and adjacent sequence because deleted forms of Rx that lack constitutive gain of function mutations are not active unless the protein is overexpressed.

Keywords: potato virus X; coat protein; hypersensitive response; cell death.

Introduction

Many disease resistance (R) genes in plants encode proteins with nucleotide binding site (NBS) and leucine rich repeat (LRR) domains (Ellis et al., 2000; Hammond-Kosack and Jones, 1997). Genetic evidence indicates that these NBS-LRR proteins are receptors that interact, directly or indirectly, with elicitors produced by the pathogen. Following recognition there is activation of downstream signalling pathways leading to disease resistance and a hypersensitive response (HR) that is manifested as cell death (Shirasu and Schulze-Lefer, 2000).

In common with other receptors it is generally considered that NBS-LRR proteins have a modular structure with separate recognition and signalling domains. The LRR is a candidate recognition domain because it is the most variable region in closely related NBS-LRR proteins and is under selection to diverge (Meyers et al., 1998; Noel et al., 1999). Functional analysis of recombinant R proteins also indicates that recognition specificity resides in the LRR (Ellis et al., 1999). However, there is indirect evidence that the LRR can contribute to signalling as well as recognition. (Banerjee et al., 2001; Warren et al., 1998)

The amino terminal region in NBS-LRR R proteins is thought to be the major signalling domain. In most NBS-LRR proteins this region includes either a Toll-interleukin receptor (TIR)-like domain or a putative coiled-coil (CC) structure (Ellis et al., 2000). In Arabidopsis, TIR-NBS-LRR proteins apparently signal through the EDS1 lipase-like protein, whereas CC-NBS-LRR proteins require pathways involving NDR1 (Aarts et al., 1998). RPS7 and RPP8 are NBS-LRR proteins but do not have absolute dependency on EDS1 or NDR1 and it is thought that they signal through a novel, as yet uncharacterized, pathway (McDowell et al., 2000). In some NBS-LRR proteins the amino terminal domain may be involved in recognition as well as signalling. For example, from the novel resistance specificity of recombinant L6, L2 and LH flax rust R genes, it seems likely
that recognition can be influenced by a TIR domain (Ellis et al., 1999; Luck et al., 2000).

The NBS is positioned between the putative signalling domain and the LRR. Its precise function is not known but there are clues from sequence motifs that are also present in the NBS of CED-4 (C. elegans) and APAF-1 (human) regulators of apoptosis (Aravind et al., 1999; van der Biezen and Jones, 1998). The common sequences include the P loop (kinase 1), kinase 2 and kinase 3a and other motifs found in ATPase proteins from both prokaryotes and eukaryotes (Aravind et al., 1999). Mutation analysis indicates that at least some of these motifs are required for the function of R proteins (Axtell et al., 2001; Dinesh-Kumar et al., 2000; Tao et al., 2000; Tornero et al., 2002). By analogy to APAF-1 and similar proteins, it seems likely that ATPase activity in NBS-LRR proteins mediates conformational changes necessary for downstream signalling and disease resistance (Hu et al., 1999).

Indirect evidence from the nematode resistance gene Mi indicates how the activity of NBS-LRR proteins could be controlled (Hwang et al., 2000). Recombinant Mi proteins containing elements of a non-functional Mi homologue activate an HR in the absence of elicitor. To explain this result it was proposed that there are intra-molecular interactions between domains of Mi. In the wild-type protein in the absence of elicitor these interactions would prevent signalling of disease resistance and cell death. Modification of the interaction, either following elicitor recognition or by mismatch of interacting residues in the recombinant proteins, would allow the signalling domains of Mi to activate the response pathways. The lesion mimic phenotype of a natural recombinant of the Rp1 resistance gene in maize (Sun et al., 2001) is also consistent with this interpretation.

Here we investigate structure-function relationships in the CC-NBS-LRR protein encoded by Rx. Rx confers resistance against Potato virus X (PVX) in potato (Cockerham, 1970) and the elicitor is the viral coat protein (CP) (Bendahmane et al., 1995). Rx-mediated resistance is unusual in that it is not normally associated with an HR either in potato or as a transgene in Nicotiana species (Bendahmane et al., 1999). However transgenic expression of the CP elicits an Rx-dependent HR in potato and in Nicotiana species (Bendahmane et al., 1999). Our approach to the analysis of Rx involved screening for Rx mutants that mediate an HR in the absence of the elicitor CP. We also tested the over-expression of wild type and deleted forms of Rx for an elicitor-independent HR. Our results imply that conserved motifs in the NBS and LRR regions of Rx regulate a signalling domain in the amino terminal region. As deletion or mutation of these motifs leads to gain of Rx function it is likely that they have an inhibitory role in the wild-type protein. Presumably this inhibitory function is abrogated in the presence of the coat protein elicitor.

Results

Random mutagenesis of Rx

To address the possible role of the NBS and other domains in Rx we screened for Rx mutants in which an HR was activated in the absence of the elicitor PVX CP. The mutants were generated by PCR of Rx cDNA under conditions leading to misincorporation of nucleotides. The PCR products were then inserted between the promoter and transcriptional terminator of Rx (Figure 1a, pR-AT) and the constructs were assembled in the T-DNA region of an Agrobacterium binary plasmid vector. A library of Rx mutants was generated and individual clones were assayed for HR induction by infiltration of liquid Agrobacterium cultures into leaves of Nicotiana tabacum. All experiments were repeated at least three times with several replicates in each experiment and the data, as presented here, were consistent and reproducible in all instances.

Agrobacterium cultures carrying a wild-type Rx cDNA construct (pR-Rx; Figure 1a) did not induce an HR when infiltrated into non-transformed N. tabacum leaves. However, when this construct was infiltrated into transgenic N. tabacum producing the CP of PVX (Spillane et al., 1997), there was necrosis after 48 h and death of the infiltrated region by 72 h (Figure 1b). Presumably the transiently expressed Rx had recognized the elicitor CP and activated signalling leading to cell death.

Out of 2500 mutant Rx clones tested there were seven that induced an HR in the leaves of non-transformed N. tabacum. Figure 1(b) illustrates the HR phenotype of one of these constitutive gain-of-function mutants (AT25). Four of the mutants (AT39, AT193, AT25, and AT7) induced an HR within 48 h of Agrobacterium infiltration. The others (AT32, AT72 and AT28) induced an HR that was delayed until 48–72 h post infiltration. These gain-of-function mutants all showed an HR in the presence of the coat protein (Figure 1b).

Constitutive gain-of-function mutants are modified in the NBS and LRR domains

DNA sequence analysis revealed that each of the constitutive gain-of-function forms of Rx carries between three and 11 amino acid substitutions relative to the wild type. To identify the amino acid substitutions implicated in the activation of Rx we constructed a series of recombinant molecules incorporating elements of the wild type and constitutive gain-of-function mutants. For each of the gain-of-function mutants we identified recombinant molecules with a single amino acid mutation that induced an elicitor-independent HR on non-transformed N. tabacum using the Agrobacterium infiltration assay.

In each of the pR-AT39, pR-AT193, pR-AT25, pR-AT32, pR-AT72 and pR-AT28 clones a single mutation was
responsible for the HR (Figure 2a). In each instance the timing of the HR was the same with the single amino acid mutant and the corresponding progenitor clone. The pR-AT7 clone carried two mutations that were independently responsible for activation of an HR (Figure 2a). The pR-AT7-derived recombinants with single amino acid mutations caused an HR that was slower than with the progenitor pR-AT7. Presumably the faster HR of the progenitor pR-AT7 was due to an additive effect of the two mutations.

Three out of these eight mutations (F393I, D399V, E400K) are in an eight amino acid interval close to the RNBS-D (CFLY) motif that is conserved in R proteins (Hammond-Kosack and Jones, 1997; Meyers et al., 1999). Two other mutations led to the same amino acid change (D460V) in the conserved MHD motif (Hammond-Kosack and Jones, 1997) (Figure 2a,b) but are derived independently because, in the

**Figure 1.** Induction of HR by a constitutive gain-of-function mutant of Rx. (a) Schematic representation of T-DNA constructs for expression of Rx cDNA. The cDNA inserts of wild-type or mutant forms of Rx were inserted between Rx promoter (pR) and transcriptional terminator (ter). The black box indicates the cDNA or either wild-type (wt) Rx cDNA or the cDNA of mutant (AT) forms of Rx. LB and RB indicate the left and right border of the T-DNA. (b) Expression of wild type Rx and the constitutive gain-of-function mutant pR-AT25 in N. tabacum. The Rx constructs were the wild type Rx (pR-Rx) or the constitutive gain-of-function mutant (pR-AT25). Agrobacterium cultures carrying pR-Rx or pR-AT25 was infiltrated into leaves of either non-transformed (NT) or transgenic N. tabacum (CP) expressing the PVX CP from the 35S promoter. The leaves were photographed 4 days after infiltration.

**Figure 2.** The sequence of the Rx constitutive gain-of-function mutants. (a) Sequence changes responsible for constitutive gain-of-function phenotypes. The diagram shows the changes at the nucleotide and protein level that are responsible for the constitutive gain-of-function phenotype. (b) The predicted protein sequence of Rx. The sequence is divided into three domains corresponding to the amino terminal CC domain, the NBS domain and the LRR. The LRR consensus motifs are shown in blue and are aligned. The conserved VLDL motif in LRR3 is underlined.
The Rx gain-of-function phenotype is dependent on a wild-type P loop motif and on SGT1

In principle the constitutive gain-of-function mutants could elicit an HR because the encoded proteins are toxic in the Agrobacterium-infiltrated leaves. Alternatively these mutant proteins could have activated the downstream pathway that is involved in the Rx-mediated response to the PVX CP. To test these possibilities we introduced alanine substitutions (G175A + K176A) in the P loop (K1) motif of the NBS (Aravind et al., 1999) (Figure 2) and assayed the HR phenotype with the Agrobacterium infiltration assay in non-transgenic and CP transgenic plants. We predicted that an HR related to Rx-mediated resistance would depend on integrity of the conserved P loop, whereas an HR due to toxic effects of protein over-expression in the Agrobacterium infiltration assay would be unaffected by disruption of the NBS.

As predicted the P loop Rx mutants failed to show an HR in the infiltrated region of the CP transgenic plants (Figure 3a) indicating that activation of downstream signalling requires integrity of the NBS. Similarly, when the P loop mutation was introduced into Rx gain-of-function mutants, there was no HR in non-transformed N. tabacum leaves. Identical results were obtained with HA epitope tagged versions of Rx and the constitutive gain-of-function mutants. Thus, Rx(HA) was fully functional in an Agrobacterium infiltration HR assay in the CP transgenic N. tabacum and was readily detected by Western blot analysis with HA antibody in non-transgenic plants (Figure 3b). The P loop mutants of Rx(HA), D543E(HA) and D460V(HA) were also non-functional in the HR assay although the encoded proteins were detected by Western analysis (Figure 3b). From these results we can rule out that the lack of an HR with the P loop mutants was due to protein destabilization. A more likely explanation is that the P loop has the same function in wild type and gain-of-function mutants of Rx. This interpretation is reinforced by our finding (AB, data not shown) that mutations in residues D244A + D245A (kinase 2) or G330A + P332A (GLPL) abrogated the ability of both wild-type and constitutive gain-of-function mutants to produce an HR in the infiltration assay.

Further indication that the gain-of-function phenotype is relevant to Rx-mediated resistance was based on an HR assay in N. benthamiana following virus induced gene silencing (VIGS) of SGT1 loci. SGT1 proteins are required for many types of disease resistance responses (Austin et al., 2002; Azevedo et al., 2002) including the HR associated with Rx (Figure 3c)(Peart et al., 2002). However expression of SGT1 loci is not required for cell death induced by ethanol, sodium azide or sodium chloride (Peart et al., 2002). Based on these findings we predicted that the constitutive gain-of-function HR would only be compromised by silencing of SGT1 if it were a bona fide resistance response. A toxic response would not be compromised by SGT1 silencing. The HR tests in SGT1 silenced plants were carried out with D460V and H519R + D543E + H738R in which three LRR gain-of-function mutations were combined in one construct. The SGT1 silencing was induced by infection of N. benthamiana with a tobacco rattle virus vector (Ratcliff et al., 2001) construct carrying an SGT1 cDNA insert.

The results shown in Figure 3(c) show that SGT1 expression is required for an HR induced by the mutant Rx proteins. This requirement is not because SGT1 silencing causes reduced levels of Rx (P. Moffett, unpublished data). It is likely therefore that the constitutive gain-of-function phenotype represents Rx-mediated resistance rather than a toxic response. The SGT1 requirement for other gain-of-function mutants could not be tested because, for reasons that we do not understand, they did not produce an elicitor-independent HR in N. benthamiana unless they accumulate at very high levels due to co-expression in the presence of a suppressor of gene silencing (A. Bendahmane, G. Farnham and P. Moffett unpublished observations). In our hands virus-induced silencing is much more effective in N. benthamiana than in N. tabacum (Ana Montserrat Martin Hernandez, unpublished observations).

Overexpression of Rx

As a second approach to structure-function analysis of Rx we used the Agrobacterium infiltration assay to overexpress full length and deleted Rx constructs in the absence of elicitor (Figure 4a). We predicted that overexpression of the full length Rx in N. tabacum would activate resistance in the absence of elicitor, as with other NBS-LRR proteins (Oldroyd and Staskawicz, 1998; Tao et al., 2000). We also predicted that deletion of domains required for downstream signalling would prevent the overexpression phenotype. However, deletions in inhibitory domains would either have no effect or would enhance the overexpression phenotype. The overexpression constructs all included a carboxy terminal HA tag so that the level of the Rx proteins could be monitored by immuno-blotting with HA antibody.

As predicted, expression of full length Rx(HA) under the strong constitutive 35S RNA promoter of CaMV (Figure 4a) induced an HR that developed by 72 h post infiltration in the absence of PVX coat protein. The same protein, either with or without the HA tag did not induce an HR when expressed...
from the genomic Rx promoter (Figure 1b). The level of Rx(HA) was higher from the 35S construct than with the Rx promoter (Figure 4b) and we conclude that the HR is caused by overexpression of Rx.

Overexpression of three deleted versions of Rx(HA) (382Δ, 318Δ and 293Δ) resulted in an HR that developed more rapidly (< 48 h) than with the full length protein (72 h). The 282Δ protein induced a very slow HR (> 96 h) and the 1Δ138 amino terminal deletant was inactive. These deleted proteins were HA tagged but we could not detect the 382Δ, 318Δ and 293Δ proteins in N. tabacum because rapid HR occurred before they had accumulated to detectable levels.

Figure 3. Constitutive gain-of-function phenotypes require P loop-dependent signalling and SGT1.
(a) Rx constructs were infiltrated into N. tabacum that was either non-transgenic or that expressed the PVX CP. Production of an HR was monitored over a period of 4 days. The Rx constructs were either in a wild-type background or with constitutive gain-of-function mutations E440K, D460V or H519R + D543E + H738R. The P loop motif in these constructs was either wild type or had GK residues at position 175 and 176 modified to AA (K1). HR indicates an HR within 48 h of infiltration and ‘-’ indicates no HR.
(b) Western blot analysis of HA tagged versions of Rx and constitutive gain-of-function mutants with P loop (K1) mutations. The constructs were either the wild-type Rx with an HA tag (RxHA), K1 mutant Rx with an HA tag (RxHAK1) and RxHA constructs with double constitutive gain-of-function and K1 mutations (D543E)K1 and (D460V)K1). The constructs were transiently expressed using Agrobacterium infiltration (O.D.600 = 0.2). Samples were taken at 3 days post infiltration, fractionated by SDS-PAGE and the HA tagged proteins were detected with anti-HA antibody. The HR or ‘-’ symbols indicate whether the proteins were able to mediate an HR within 48 h either in the presence or absence of CP.
(c) N. benthamiana plants were infected with TRV:00 or TRV:SGT to silence SGT1 expression. After 21d Agrobacterium infiltration was used to introduce Rx constructs into the upper infected leaves of the plants either individually or in combination with constructs carrying a 35S CP construct. The PVX constructs were either wild type Rx in the presence (1) or absence (2) of CP, D460V (3) or H519R + D543E + H738R (4). The images show that Rx mediated an HR in TRV:00-infected plants either as a wild-type Rx in the presence of the CP construct or as a constitutive gain-of-function mutant (right leaf) but that SGT1 silencing compromised the resistance-associated HR (left leaf).

However in *N. benthamiana* none of these proteins induced an HR and they could be detected at 48 h post infiltration by western analysis (Figure 4c). All of the deleted forms of Rx were substantially less abundant than full length Rx. Based on the assumption that accumulation in *N. benthamiana* is indicative of levels in *N. tabacum* these results show that HR-inducing activity is enhanced if the carboxy terminal part of the protein is removed. Thus 382Δ, 318Δ and 293Δ elicited a rapid HR in *N. tabacum*, despite their accumulation at a lower level than the full length protein. This interpretation is consistent with the proposal, based on the gain-of-function mutant phenotype described above, that there are negative regulatory functions in Rx between residues 382 and the carboxy terminus.

The removal of sequence on the amino terminal side of residue 293 either reduced (282Δ) or abolished (1Δ138) HR-inducing activity, even though these proteins were at least as abundant as those with a rapid HR phenotype (Figure 4c). The 282Δ construct differs from the 293Δ construct that elicited a more rapid HR in that it lacks a complete kinase 3 motif and the completely inactive 1Δ138 is deleted in the CC domain. It is likely therefore that the CC domain and the kinase 3 motif of the NBS are essential for downstream signaling capability of Rx.

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**Figure 4.** Over expression of Rx deletion mutants.

(a) A scale diagram of Rx showing the CC and LRR regions, as in Figure 2. In the NBS domain the locations are shown of the P loop(K1), kinase 2 kinase 3a and other motifs that are conserved in NBS-LRR proteins including APAF-1 and CED4. The lines indicate the extent of deletions in HA tagged constructs relative to these conserved motifs. The figures in the right hand side column indicate the timing of an HR with these constructs when transiently expressed from a 35S promoter in the absence of CP (NR = no response).

(b) Western analysis of Rx HA expressed in *N. tabacum* in an Agrobacterium transient assay (O.D. 600 = 0.2) either from the Rx promoter (pB1) or from the 35S promoter (35S). The Rx constructs encoded either the wild-type protein of the P loop (K1) mutants. Protein extracts were prepared 40 h post-infiltration, prior to the onset of HR, separated by SDS-PAGE and detected by anti-HA immuno-blotting. An asterisk denotes that in these samples, five-fold less protein extract was loaded.

(c) Western analysis of Rx HA and deletion constructs expressed in an Agrobacterium transient assay (O.D. 600 = 0.5) from the 35S promoter of CaMV in *N. benthamiana*. Protein extracts from duplicate experiments were prepared 48 h post-infiltration, separated by SDS-PAGE and detected by anti-HA immuno-blotting.
**Discussion**

**Regulatory domains in Rx and other NBS-LRR proteins**

In this paper we report two findings related to the function of Rx and, presumably, other NBS-LRR proteins. First we describe constitutive gain-of-function mutations in the central and carboxy terminal regions of the Rx protein and, second, we show that an HR induced by overexpression of Rx can be accelerated by deletion of conserved sequence motifs in the NBS. We conclude that these Rx phenotypes are related to signalling rather than recognition because they involve an elicitor-independent HR.

In one scenario a constitutive gain-of-function mutation would have fortuitously generated specific structures of Rx that normally exist only in the presence of elicitor. Alternatively, the gain-of-function mutations could have an indirect effect by, for example, disruption of inhibitory domains in Rx that prevent signalling in the absence of elicitor. Formally, from the data presented here, neither interpretation can be ruled out. However it is unlikely that random mutagenesis would repeatedly generate new specific structures involved in downstream signalling. A more likely scenario is that the mutation has a disruptive effect and that the constitutive gain-of-function mutations affect inhibitory domains in Rx. A similar interpretation, invoking inactivation of inhibitory domains, was used to explain a constitutive gain-of-function phenotype in a mutant Pto protein (Rathjen et al., 1999).

Other NBS-LRR proteins that, like Rx, may be subject to negative regulation include Nod1 in humans. This protein exhibits enhanced signalling activity when over-expressed with a deletion in the LRR (Inohara et al., 1999) and it was concluded that the LRR prevents signalling by amino terminal domains. The analysis of the nematode resistance protein Mi is also consistent with negative regulatory domains in NBS-LRR proteins. Recombinants of Mi and an inactive homologue induced an elicitor-independent HR (Hwang et al., 2000) like the constitutive gain of function Rx mutants described here. To explain this phenotype, it was proposed that there are intramolecular interactions in Mi (Hwang et al., 2000). In the recombinants or, presumably in the presence of the Mi elicitor, these inhibitory interactions would be disrupted so that signalling domains would become exposed and available for interaction with components of the downstream pathway.

**Conserved sequence motifs in NBS-LRR proteins**

Most of the constitutive gain-of-function mutations in Rx are in or close to sequence motifs that are common to many NBS-LRR proteins. For example the D543E (Figure 2) mutation is in the VLDL motif that is present in the third LRR of many NBS-LRR proteins (Axtell et al., 2001) (G. Farnham, unpublished data) including RPS5 in Arabidopsis. RPS5 encodes an NBS-LRR protein and confers resistance against Pseudomonas syringae. However a VLDL motif mutant allele, rps5-1, has a complex phenotype involving loss of RPS5-mediated resistance and suppression of resistance conferred by other NBS-LRR proteins (Warren et al., 1998).

To reconcile the interference phenotype of rps5-1 and the constitutive gain-of-function of D543E we propose that these mutations both disrupt a negative regulatory domain in the respective NBS-LRR proteins. Complete inactivation of this domain, as in D543E, would release the negative regulation so that the Rx protein could interact with downstream components of the signalling pathway and activate an HR in the absence of elicitor. Partial inactivation of this domain could allow the NBS-LRR protein to interact with downstream signalling components but, as may be the case with rps5-1, without activation of signalling. In that scenario, if the downstream signalling components are required by other NBS-LRR proteins, a partial loss-of-function would interfere with the respective signalling pathways.

Other NBS-LRR motifs, including RNBS-D (Meyers et al., 1999) and MHD (van der Biezen and Jones, 1998) are implicated in Rx inhibitory domains by constitutive gain-of-function mutant phenotypes of D399V, E400K and D460V (Figure 2). The MHD motif had not been targeted previously in mutational analysis of NBS-LRR proteins (Axtell et al., 2001; Dinesh-Kumar et al., 2000; Tao et al., 2000; Tornero et al., 2002) and so the data presented here provide the first direct analysis of its function.

Other than Rx, the most extensively mutagenised NBS-LRR protein is RPM1 (Tornero et al., 2002). A total of 53 missense loss-of-function alleles were identified at this locus of which only one is at or adjacent to the conserved motifs that were modified in the Rx gain-of-function alleles. The one exception (rpm1-12) affected the RNBS-D motif that was modified in the AT72 and AT25 alleles of Rx (Figure 2). However the mutated amino acid in rpm1-12 is not conserved in Rx and therefore is not directly comparable with our constitutive gain-of-function alleles. The other RPM1 mutants, like the gain-of-function mutants of Rx, were predominantly either within or on the carboxy terminal side of the NBS. However, it is not possible to draw additional conclusions by cross referencing between the two sets of data because the gain- or loss-of-function screens would identify different functional domains.

**Amino terminal domains**

Our constitutive gain-of-function mutants are not directly informative about signalling domains in Rx. However, since the HR would require signalling, the phenotypes of the Rx deletion mutants (1.A138, 382Δ, 318Δ and 293Δ; Figure 4) are...
indirectly informative. They indicate, consistent with previous suggestions (Aarts et al., 1998), that signalling involves the amino terminal domain in combination with the P loop, kinase 2 and kinase 3a motifs. It is striking that none of the constitutive gain-of-function mutations was within this region of the Rx protein. Perhaps, as suggested above, random mutagenesis leads primarily to disruption of functional domains rather than the generation of specific structures that are required for interaction with components of the signalling pathway.

A gain of function analysis of the nematode resistance gene Mi led to the conclusion that the amino terminal domain was a regulator of signalling mediated by the LRR. This interpretation differs from that made here about Rx and it may be that activation of Mi- and Rx-mediated resistance involves different mechanisms. However, if as seems likely, the NBS-LRR domains have multiple functions in recognition, activation and response signalling these two sets of data are not necessarily incompatible. The artificial Mi recombinants would have been informative about some but not all of these functions. Similarly the Rx point mutants would have provided information about a different set of functions. For more complete understanding of recognition and response in disease resistance, it will be necessary to generate high resolution secondary and tertiary structures of Rx and other NBS-LRR proteins and their elicitors.

Experimental procedures

Rx constructs

pB1 is a modified pBIN19 plasmid (Bevan, 1984) that carries a transcription cassette comprising 3 kb of the Rx promoter and a 1.5-kb Rx terminator separated by an XbaI and a SacI cloning sites (Bendahmane et al., 2000). All Rx derivative mutants were cloned between the XbaI and the SacI cloning sites. All constructs were sequenced to confirm the absence of PCR-derived mutations. To construct pR-Rx, Rx cDNA was PCR amplified and the PCR product was cloned into the XbaI and SacI sites of pB1 to create pR-Rx.

The 35S promoter constructs were all in the pBIN1 binary vector. pBIN1 is a modified pBIN19 binary vector that carries a transcription cassette comprising the CaMV 35S promoter and terminator. To construct the pBIN1 binary vector, the transcription cassette containing the CaMV 35S promoter and terminator was released by digestion with KpnI and XhoI from the plasmid pJIT61 (kindly provided by P. Mullineaux, JIC, Norwich, UK). The transcription cassette was then ligated to the pBIN19 plasmid vector digested with KpnI and SalI to create pBIN1. To construct 35S-Rx, Rx cDNA was PCR amplified and cloned into the XbaI and SalI sites of pBIN1.

RxHA was constructed via PCR, using a primer that introduced a BamHI site at the extreme carboxyl terminus of the Rx open reading frame followed by the HA epitope tag. Similarly the truncated constructs were generated by PCR from the Rx cDNA with 3’ primers introducing a BamHI site. The PCR products were cloned into the XbaI/BamHI sites of PBin61-RxHA and subsequently transferred to pB1.

Full details of all constructs are available on request and biomaterials are available through our website (http://www.sainsbury-laboratory.ac.uk).

PCR mutagenesis

Random mutagenesis of the Rx gene was performed under conditions similar to those previously described (Shafik Khan et al., 1997). The PCR was carried out using the primers RxP1 and Rxac4, which flank the Rx ORF. The PCR reaction contained (100 μl final volume) 10 mM Tris (pH 8.3), 50 mM KCl, 0.05% Nonidet P-40, 7 mM MgCl2, 0.15 mM MnCl2, 0.2 mM dGTP, 0.2 mM dATP, 1 mM dCTP, 1 mM dTTP, 0.3 μM of both primers, 50 ng of template and 5 U Taq DNA polymerase (Gibco-BRL). PCR was performed for 35 cycles: 15 s at 94°C, 15 s at 55°C, and 2 min at 72°C. The PCR products were digested with XbaI and SacI, gel purified and cloned in the binary vector pB1 in E. coli. Plasmid DNA was purified from 10000 colonies and electroporated into A. tumefaciens strain C58C1 carrying the virulence helper plasmid pCH32 (Hamilton et al., 1996).

Site-directed mutagenesis

Oligonucleotide-directed mutagenesis was used to introduce specific mutations into the Rx cDNA or into the mutants E400K, D460V and D543E + H738R. The site directed mutations were confirmed by sequence analysis. The mutations of the P loop were at Rx codon 175 from GGG(G) to GCG(A) and at codon 176 from AAA(K) to GCA(A). The mutations of the kinase Za motif were at Rx codon 244 from GAT(D) to GCT(A) and at codon 245 from GAC(D) to GCC(A). The mutations of the GLPL motif were at codons 330 from GGA(G) to GCA(A) and 332 from CCT(P) to GCT(A).

Agrobacterium-mediated transient expression

Agrobacterium-mediated transient expression was performed under conditions similar to those described previously (Bendahmane et al., 1999). The binary Ti-plasmid vector constructs were transformed into A. tumefaciens strain C58C1 carrying the virulence helper plasmid pCH32 (Hamilton et al., 1996). The transformants were inoculated into 5 ml L-broth medium supplemented with 50 μg ml⁻¹ kanamycin and 5 μg ml⁻¹ tetracycline and grown at 28°C overnight. Cells were precipitated and resuspended to the OD of 0.5 in solution containing 10 mM MgCl2, 10 mM MES pH 5.6 and 150 μM acetylsyringone. The cells were left at room temperature on the bench for 2 h before infiltration into N. tabacum leaves. The infiltrations were either into non-transformed N. tabacum or into transgenic N. tabacum expressing the PVX CP (Spillane et al., 1997). The transient expression was more efficient in the first true leaf of young N. tabacum seedlings.

DNA sequencing and analysis

The sequencing reactions were performed using a dye terminator cycle sequencing reaction kit (Perkin-Elmer, Cambridge, UK). Sequence reactions were resolved on ABI377 automated sequencer (Applied Biosystems ABI, La Jolla, CA, USA). Sequence contigs were assembled using UNIX versions of the Staden programs package (Staden, 1996).

Immunodetection

To prepare protein samples, two leaf discs (approximately 35 mg) were ground in 150 μl of 8 M urea, followed by addition of 75 μl of

3X SDS loading buffer and boiling for 10 min Fifty μl of protein extract was separated by 10% SDS-PAGE followed by transfer to Immun-blot PVDF membrane (Bio-Rad, Herem Hempstead, UK). Membranes were incubated successively in 5% skim milk powder in TBST (25 mM Tris–HCl (pH 7.5), 150 mM NaCl, 0.1% Tween-20, 0.1 ng ml⁻¹ of 3F10 anti-HA antibody (Roche, Lewes, UK) and finally with goat anti rat HRP conjugate (Sigma, Poole, UK). Proteins were visualised using the chemiluminesence kit ECL Plus (Pharmacia, Little Chalfont, UK).

**References**


