Research Note

Elicitation of *Rx*-Mediated Resistance to PVX in Potato Does Not Require New RNA Synthesis and May Involve a Latent Hypersensitive Response

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Accepted 5 May 1998.

The *Rx* gene in potato confers extreme resistance against potato virus X (PVX) that is elicited by the viral coat protein (CP). Using actinomycin D to inhibit transcription of nuclear genes, we have shown that *Rx* is expressed prior to virus inoculation and that induction of the resistance mechanism does not require novel gene expression or continued transcription of previously expressed genes. We have also shown that transgenic plants could not be produced carrying a CP transgene from an *Rx*-avirulent PVX in an *Rx* background. These data indicate that *Rx*-mediated resistance involves a mechanism that suppresses viability of plant cells.

Additional keywords: cell death, disease resistance, protoplasts.

The *Rx* locus in potato confers an extreme resistance to potato virus X (PVX) (Goulden et al. 1993; Köhm et al. 1993). In natural conditions the resistance is specific for PVX (Goulden et al. 1993). However, by experimental co-inoculation of PVX and a second virus, it has been shown that the *Rx* resistance mechanism has the potential to suppress accumulation of viruses unrelated to PVX in protoplasts (Köhm et al. 1993; Bendahmane et al. 1995). We inferred from these co-inoculation experiments that there are separate recognition and response phases in the resistance mechanism. The recognition phase would involve the interaction of a PVX-encoded elicitor with a hypothetical receptor molecule encoded in the plant. It has now been established that the PVX coat protein (CP) is the elicitor of *Rx*-mediated resistance (Bendahmane et al. 1995). The receptor is likely to be the product of *Rx*.

The separate recognition and response phases of *Rx*-mediated resistance are characteristic of several disease resistance mechanisms in plants (Dangl 1995). However, unusual among resistance responses in plants, there is no cell death (hypersensitive response; HR) (Godiard et al. 1994) associ-

ated with *Rx*-mediated resistance. Two reasons were proposed previously as an explanation for the absence of an HR (Goulden and Baulcombe 1993; Köhm et al. 1993). It could be that the *Rx* does not activate cellular responses leading to an HR. Alternatively, an HR may be a slow secondary response associated with *Rx*-mediated resistance. In this latter scenario the fast primary *Rx* response would keep accumulation of PVX below the level required for elicitation of the secondary resistance responses and the HR would remain latent.

To investigate the nature of *Rx*-mediated resistance, a transgene encoding the PVX CP elicitor of *Rx* was introduced into various potato cultivars. Based on related studies (Culver and Dawson 1991; Jones et al. 1994) it was predicted that transgenic expression of the elicitor would elicit a necrotic phenotype if there is the potential for hypersensitivity as a secondary *Rx* response. We have also investigated the requirement for plant RNA synthesis in the elicitation of *Rx*-mediated resistance.

The effect of actinomycin D on *Rx*-mediated resistance.

In previous analyses we have established that PVX accumulation in protoplasts of potato cv. Cara (*Rx* genotype) is initially at the same rate as in protoplasts of cv. Maris Bard (*rx* genotype) (Köhm et al. 1993). The suppression of PVX RNA accumulation due to *Rx*-mediated resistance occurred only after a lag of several hours post inoculation. To determine whether this delay was to allow activation of host gene expression we have repeated the analyses of PVX RNA accumulation with potato protoplasts incubated in the presence of actinomycin D (AMD) at 120 µg ml⁻¹. In trial experiments it was established that this level of AMD suppressed more than 95% of ³H uridine incorporation into total or polyadenylated RNA (Table 1) over a 24-h period. Despite this substantial inhibition of DNA-dependent RNA synthesis there was no effect of AMD on the differential synthesis and accumulation of PVX_{UK3} RNA in protoplasts of potato cvs. Cara (*Rx* genotype) or Maris Bard (*rx* genotype). (Fig. 1). Based on these data, it seems likely that replication of PVX and activation of the resistance mechanism are independent of host RNA synthesis. Presumably the activation of *Rx*-mediated resistance involves plant components that were produced prior to inoculation of the protoplasts.

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Introduction of an *Rx*-elicitor CP into *Rx*-genotype potato.

Production of the PVX CP is necessary and, of the PVX-encoded proteins, sufficient for elicitation of *Rx*-mediated resistance in protoplasts (Bendahmane et al. 1995). Based on this finding, it was predicted that transgenic expression of an *Rx*-elicitor CP in Cara (*Rx* genotype) would activate *Rx*-mediated resistance in the absence of PVX. If there is a secondary HR associated with *Rx*-mediated resistance it was anticipated that these transgenic plants would exhibit a necrotic phenotype. To test these predictions the potato cvs. Cara (*Rx* genotype) and Maris Bard (*rx* genotype) were transformed (Kumar 1996) with transgene constructs based on the CP open reading frame (ORF) from various natural or mutant isolates of PVX.

In the first series of transformations the constructs were based on the PVX_{UK3} and PVX_{HB} isolates. The CP of PVX_{UK3}

Table 1. The effect of actinomycin D (AMD) on incorporation of ³H uridine into RNA of potato protoplasts^a

RNA	AMD (μg ml ⁻¹)	³ H uridine (cpm) per 2.10 ⁶ protoplasts	
		Experiment 1	Experiment 2
poly (A)+	0	9,619	2,710
poly (A)+	120	666 (7%)	95 (3.5%)
total RNA	0	172,925	59,207
total RNA	120	19,317 (11%)	2,903 (4.9%)

^a Protoplasts were incubated for 24 h in medium containing ³H uridine (35 to 50 Ci mmol⁻¹) in either presence or absence of AMD at 120 μg ml⁻¹. Incorporation into RNA was determined by binding to DE81 filters corrected for radioactivity bound to filters in extracts of protoplasts processed immediately after addition of the ³H uridine. Each value is mean of two assays with separate protoplast samples. Values in parentheses indicate residual incorporation after incubation in presence of AMD. Analyses were either of labeled total RNA isolated from 5.10⁵ protoplasts or of polyadenylated RNA of 2.10⁶ protoplasts isolated with an oligo(dT₂₅) bead purification kit from Dynal (Bromborough, UK).

Fig. 1. Host RNA synthesis is not required for *Rx*-mediated resistance to potato virus X (PVX). RNA was isolated from protoplasts of potato cvs. Cara (*Rx* genotype) or Maris Bard (*rx* genotype) at 24 h post inoculation by electroporation with 4 μg of RNA isolated from virus particles of PVX_{UK3} and analyzed by Northern (RNA) analysis with a PVX-specific probe (Chapman et al. 1992). The RNA inoculum was PVX_{UK3} and the protoplasts were incubated in the presence or absence of actinomycin D (AMD) (shown as + and – respectively, in white). Data are from duplicate samples for each experimental treatment. The major RNA species in the PVX-infected cells were the genomic (6.4 kb) and subgenomic (2.1 and 0.9 kb) RNAs. Lane labeled “v” was loaded with PVX virion RNA. Analysis of RNA from mock-inoculated protoplasts (m) revealed a minor RNA species migrating between the subgenomic RNAs that was a hybridization artefact of the PVX probe.

is an elicitor of *Rx*-mediated resistance whereas the CP of PVX_{HB} is not. The constructs had the respective CP cDNAs coupled to the 35S promoter of cauliflower mosaic virus in an *Agrobacterium tumefaciens* binary vectors (pSLJ4654A) (Jones et al. 1992). The Maris Bard (*rx* genotype) was readily transformable and from 100 explants treated with the respective *A. tumefaciens* strains we obtained six lines carrying the PVX_{UK3} transgene and six with the PVX_{HB} constructs. Polymerase chain reaction (PCR) tests confirmed the presence of the CP transgene in those lines (data not shown). Similarly, Cara (*Rx* genotype) could be transformed with the PVX_{HB} construct (seven transformants from 100 explants). However, in 21 attempts involving 420 explants we failed to produce Cara (*Rx* genotype) transformants carrying the CP ORF of PVX_{UK3}.

In an attempt to overcome the barrier to transformation of Cara (*Rx* genotype) with CP transgenes we carried out crosses of this cultivar to Glenroe (*rx* genotype) or Maris Bard (*rx* genotype) lines carrying a CP transgene. The Glenroe parents carried constructs with the CP ORF of isolates PVX_{HB} (cross 1) or PVX_{CP4} (cross 2). The Maris Bard parent in cross 3 carried a CP transgene construct from PVX_{UK3}. Unfortunately, the CP transgenic lines available to us flowered poorly and it was possible to make only these three crosses.

The segregation of the transgene in the progeny of the crosses was assessed by Southern blot analysis with a probe specific for the CP transgene. Segregation of *Rx* was diagnosed by analysis of a PCR-amplified marker (GP34) that is tightly linked (0.5% recombination) to the *Rx1* locus carried in Cara (Bendahmane et al. 1997). In the progeny of cross 1 in which the CP transgene was from the *Rx*-breaking PVX_{HB} isolate there all four potential classes of segregant (GP34^{Rx}, CP; GP34^{Rx}, –; GP34^{Rx}, –; GP34^{Rx}, –) (Table 2). In contrast, in the progeny of cross 2 in which the CP transgene from PVX_{CP4} encoded an elicitor of *Rx*-mediated resistance, there were no segregants with the GP34^{Rx}, CP genotype (Table 2). The absence of these segregants was not due to their failure to germinate (germination rate was >95%). Nor was it due to

Table 2. Segregation analysis of coat protein (CP) transgenes and a GP34 marker of *Rx*, showing the number of representatives of each genotype in the progeny of crosses 1, 2, and 3

Progeny genotype ^a	Source of CP transgene		
	PVXHB -cross 1 ^b	PVXCP4 -cross 2 ^b	PVXUK3 -cross 3 ^b
GP34 ^{Rx} , CP	10	0	8
GP34 ^{Rx} , –	9	6	7
GP34 ^{Rx} , CP	10	10	6
GP34 ^{Rx} , –	3	10	6
Total	32	26	29

^a GP34^{Rx} indicates presence of polymorphic form of marker GP34 that is linked to *Rx*. GP34^{Rx} refers to absence of this form of the marker. For the polymerase chain reaction (PCR) analysis of the GP34 DNA marker linked to *Rx* the primers were GP34-F (CGT-TGC-TAG-GTA-AGC-ATG-AAG-AAG) and GP34-R (GTT-ATC-GTT-GAT-TTC-TCG-TTC-CG) (Bendahmane et al. 1997). PCR conditions were 94°C (1 min, 1 cycle) followed by 35 cycles of 94°C (15 s), 62°C (15 s) 72°C (1 min) and one cycle of 72°C (10 min). PCR products were digested with 0.5 U of *TaqI* (Pharmacia, Little Chalfont, U.K.) for 2 h at 65°C and an aliquot of the digest was fractionated by electrophoresis on a 2% Nusieve (Flowgen, Lichfield, U.K.) agarose gel.

^b Viral origin of CP transgenes in the pollen donor of each cross. Maternal parent for all three crosses was cultivar Cara (*Rx* genotype).

seed abortion at a late stage because the seed in the potato berries were of uniform healthy appearance (data not shown). A more likely explanation was arrest at an early stage of seed development as a consequence of the activation of *Rx*-mediated resistance by the transgenic CP of PVX_{CP4}.

In the progeny of cross 3, the GP34^{Rx}, CP genotypes were fully represented (Table 2), indicating that the CP transgene had not elicited an *Rx*-dependent response. However, Northern analysis of CP transgene revealed that the discrepancy between this result and the segregation data of cross 2 is likely due to variation in transgene expression. The CP transgene in the parent of cross 2 produced a steady-state level of CP mRNA that was readily detected by Northern analysis whereas the CP mRNA was undetectable in the transgenic parent of cross 3. Presumably, this transgene was subject to transcriptional or post-transcriptional gene silencing. In the light of this low or null expression of the transgene, the cosegregation of GP34^{Rx} and the CP transgene in the progeny of cross 3 is consistent with the proposed incompatibility of *Rx* with transgenic expression of an elicitor CP. We envisage two types of process that could result in this incompatibility of *Rx* with the CP of an *Rx*-avirulent PVX. There could be a mechanism, for example the degradation of double-stranded RNA, that suppresses virus accumulation. This mechanism, which may be similar to a mechanism of virus resistance in animal cells (Truve et al. 1993) may not have an immediate, lethal effect on mature leaf cells. Consequently, as observed, there would be no cell death on graft-inoculated plants displaying *Rx*-mediated resistance to PVX. Protoplasts in which *Rx*-mediated resistance was activated by inoculation would remain viable, as observed previously (Köhm et al. 1993). However, it might be expected that the degradation of double-stranded RNA would target secondary structure features of host RNA. As a result, the activation of the *Rx* resistance mechanism would lead to death of plant tissues at stages of growth or differentiation when RNA accumulation is rate limiting.

An alternative interpretation of the incompatibility of *Rx* with the elicitor CP is that *Rx*-mediated resistance is associated with an HR as a secondary resistance response. In inoculated cells, the extreme effectiveness of the primary resistance mechanism would prevent PVX accumulation at an early stage in the infection cycle. This arrest of virus accumulation would prevent continued expression of the virus-encoded elicitor and the secondary resistance responses would not be activated. In the transgenic plants with an *Rx* genotype the production of the elicitor CP would be uncoupled from the primary resistance mechanism. Consequently, the secondary HR would be activated and there would be death of the transformed cells.

We currently favor the explanation based on the latent HR because there are several lines of evidence to link *Rx*-mediated resistance with a necrotic response (Goulden and Baulcombe 1993; Köhm et al. 1993). These include reports of a necrotic response in *Rx* genotypes that were graft inoculated

with PVX (Benson and Hooker 1960) and the finding that an HR in *Gomphrena globosa* is elicited by the same primary structure in the PVX CP as *Rx*-mediated resistance in potato (Goulden and Baulcombe 1993).

ACKNOWLEDGMENTS

We are grateful to the Gatsby Charitable foundation for continuing support. C. S. and T. A. K. acknowledge grants from the Irish Agriculture and Food Development Agency (Teagasc), The Irish Science and Technology Agency (Forbairt), and Bioresearch Ireland. We thank George Mackay and others at SCRI for help with potato crosses and Abdel Bendahmane and Konstatin Kanyuka for comments on a draft manuscript.

LITERATURE CITED

- Bendahmane, A., Kanyuka, K. V., and Baulcombe, D. C. 1997. High resolution and physical mapping of the *Rx* gene for extreme resistance to potato virus X in tetraploid potato. *Theor. Appl. Genet.* 95:153-162.
- Bendahmane, A., Köhm, B. A., Dedi, C., and Baulcombe, D. C. 1995. The coat protein of potato virus X is a strain-specific elicitor of *Rx1*-mediated virus resistance in potato. *Plant J.* 8:933-941.
- Benson, A. P., and Hooker, W. J. 1960. Isolation of virus X from "immune" varieties of potato, *Solanum tuberosum*. *Phytopathology* 50:231-234.
- Chapman, S. N., Kavanagh, T. A., and Baulcombe, D. C. 1992. Potato virus X as a vector for gene expression in plants. *Plant J.* 2:549-557.
- Culver, J. N., and Dawson, W. O. 1991. Tobacco mosaic virus elicitor coat protein genes produce a hypersensitive phenotype in transgenic *Nicotiana sylvestris* plants. *Mol. Plant-Microbe Interact.* 4:458-463.
- Dangl, J. L. 1995. Pièce de résistance: Novel classes of plant disease resistance genes. *Cell* 80:363-366.
- Godiard, L., Grant, M. R., Dietrich, R. A., Kiedrowski, S., and Dangl, J. L. 1994. Perception and response in plant disease resistance. *Curr. Biol.* 4:662-671.
- Goulden, M. G., and Baulcombe, D. C. 1993. Functionally homologous host components recognize potato virus X in *Gomphrena globosa* and potato. *Plant Cell* 5:921-930.
- Goulden, M. G., Köhm, B. A., Santa Cruz, S., Kavanagh, T. A., and Baulcombe, D. C. 1993. A feature of the coat protein of potato virus X affects both induced virus resistance in potato and viral fitness. *Virology* 197:293-302.
- Jones, D. A., Thomas, C. M., Hammond-Kosack, K. E., Balint-Kurti, P. J., and Jones, J. D. G. 1994. Isolation of the tomato *Cf-9* gene for resistance to *Cladosporium fulvum* by transposon tagging. *Science* 266:789-793.
- Jones, J. D. G., Shlumukov, L., Carland, F., English, J. J., Scofield, S. R., Bishop, G., and Harrison, K. 1992. Effective vectors for transformation, expression of heterologous genes, and assaying transposon excision in transgenic plants. *Transgenic Res.* 1:285-297.
- Köhm, B. A., Goulden, M. G., Gilbert, J. E., Kavanagh, T. A., and Baulcombe, D. C. 1993. A potato virus X resistance gene mediates an induced, nonspecific resistance in protoplasts. *Plant Cell* 5:913-920.
- Kumar, A. 1996. Agrobacterium-mediated transformation of potato genotypes. Page 121-127 in: *Methods in Molecular Biology*. Vol. 44. M. A. Gartland and M. R. Davey, eds. Humana Press, Totowa, NJ.
- Truve, E., Aaspöllu, A., Honkanen, J., Puska, R., Mehto, M., Hassi, A., Teeri, T. H., Kelve, M., Seppänen, P., and Saarma, M. 1993. Transgenic potato plants expressing mammalian 2'-5' oligoadenylate synthetase are protected from potato virus X infection under the field conditions. *Bio/Technology* 11:1048-1052.