Molecular Plant Pathology

MOLECULAR PLANT PATHOLOGY (2016) 17(1), 120–126 DOI: 10.1111/mpp.12256

Short Communication

Evidence for different, host-dependent functioning of *Rx* against both wild-type and recombinant *Pepino mosaic virus*

CELIA R. A. DUFF-FARRIER[1], THIERRY CANDRESSE[2,3], ANDY M. BAILEY[1], NEIL BOONHAM[4] AND GARY D. FOSTER[1,*]

[1]*School of Biological Sciences, University of Bristol, Life Sciences Building, 24 Tyndall Avenue, Bristol BS8 1TQ, UK*
[2]*UMR 1332 Biologie du Fruit et Pathologie, INRA, CS 20032, 33882 Villenave d'Ornon Cedex, France*
[3]*UMR 1332 Biologie du Fruit et Pathologie, Université de Bordeaux, CS 20032, 33882 Villenave d'Ornon Cedex, France*
[4]*The Food and Environment Research Agency, Sand Hutton, York YO41 1LZ, UK*

SUMMARY

The potato *Rx* gene provides resistance against *Pepino mosaic virus* (PepMV) in tomato; however, recent work has suggested that the resistance conferred may not be durable. Resistance breaking can probably be attributed to multiple mutations observed to accumulate in the capsid protein (CP) region of resistance-breaking isolates, but this has not been confirmed through directed manipulation of an infectious PepMV clone. The present work describes the introduction of two specific mutations, A-T78 and A-T114, into the coat protein minimal elicitor region of an *Rx*-controlled PepMV isolate of the EU genotype. Enzyme-linked immunosorbent assay (ELISA) and phenotypic evaluation were conducted in three *Rx*-expressing and wild-type solanaceous hosts: *Nicotiana benthamiana*, *Nicotiana tabacum* and *Solanum lycopersicum*. Mutation A-T78 alone was sufficient to confer *Rx*-breaking activity in *N. benthamiana* and *S. lycopersicum*, whereas mutation A-T114 was found to be associated, in most cases, with a secondary A-D100 mutation to break *Rx*-mediated resistance in *S. lycopersicum*. These results suggest that the need for a second, fitness-restoring mutation may be dependent on the PepMV mutant under consideration. Both mutations conferred *Rx* breaking in *S. lycopersicum*, whereas neither conferred *Rx* breaking in *N. tabacum* and only A-T78 allowed *Rx* breaking in *N. benthamiana*, suggesting that *Rx* may function in a different manner depending on the genetic background in which it is present.

Keywords: ELISA, infectious clone, *Pepino mosaic virus*, potexvirus, resistance breaking, *Rx* gene, site-directed mutagenesis.

**Correspondence*: Email: gary.foster@bristol.ac.uk

The plant immune system is multilayered, consisting of both broad-spectrum and specific lines of defence. Dominant resistance (*R*) genes constitute an important component of these specific defence mechanisms. The products of *R* genes recognize pathogen avirulence (*Avr*) molecules and trigger a highly effective resistance response in a race-specific manner. Recognition and resistance depend on factors expressed from both the pathogen and the host, and are therefore described as a gene-for-gene interaction system (Flor, 1971). The triggered resistance commonly involves the induction of the hypersensitive response (HR) (Jones and Dangl, 2006), a form of programmed cell death resulting in necrosis at the site of infection, thereby preventing systemic viral spread.

The *Rx* gene from potato provides resistance to *Potato virus X* (PVX) in commercial potato accessions (Cockerham, 1970). It encodes a nucleotide-binding site-leucine-rich repeat (NBS-LRR)-type protein with a coiled-coil (CC) domain at the N-terminus (CC-NBS-LRR) (Bendahmane *et al*., 1999). The C-terminus of the LRR domain is thought to be involved in the specific recognition of the pathogen elicitor (Dangl and Jones, 2001; Farnham and Baulcombe, 2006). Co-expression studies have demonstrated intramolecular interactions between the CC-NBS and LRR domains to be integral in the functioning of the Rx protein. The presence of the pathogen elicitor disrupts these interactions, leading to Rx activation and defence signalling initiation (Moffett *et al*., 2002).

The resistance conferred by *Rx* is unusual in that it does not involve an HR. Viral replication has been reported to be halted in the initially infected cell and cannot therefore be detected at tissue level. For these reasons, the term 'extreme resistance' (ER) has been coined to describe it (Bendahmane *et al*., 1999; Tozzini *et al*., 1991). The PVX capsid protein (CP) is the sole elicitor of the *Rx*-based resistance response (Bendahmane *et al*., 1995, 1999; Goulden *et al*., 1993). The resistance conferred is described as durable as only a single resistance-breaking isolate is known: PVX_{HB} (Jones, 1985; Moreira *et al*., 1980). Mutational analysis has shown that the mutation of a conserved CP residue is sufficient to overcome *Rx*-mediated resistance (Goulden *et al*., 1993).

Table 1 Primers and annealing temperatures used in this investigation.

Primer number	Sequence (5'–3')	Annealing temperature (°C)
1	CAATCAACTTCTCCCCTTGGAACGG	58
2	CTCACTATAGGGAATATTAAGCTTGGTACCAATTGGTACCACGCGTTTTTTTTTTTTTTTTTTTTTTTTTT	58
3	AATGAGACTGGTCCGACCATGTGGGATCTAG	57
4	CTAGATCCCACATGGTCGGACCAGTCTCATT	57
5	ATCACGCCGAGCCCTTGCTACTCAGTTTGATCGAATCAAT	57
6	ATTGATTCGATCAAACTGAGTAGCAAGGGCTCGGCGTGAT	57

However, PVX isolates carrying this mutation are severely affected in their ability to mount a systemic infection in potato. A second, fitness-restoring mutation in the CP is necessary for full infectivity in resistant potato varieties. This need for two mutations to gain a full resistance-breaking phenotype probably accounts for the durability of *Rx* resistance (Goulden *et al*., 1993).

A number of *R* genes have been shown to retain their effectiveness when transgenically introduced into heterologous plant species (Baurès *et al*., 2008; Rommens *et al*., 1995; Song *et al*., 2003; Spassova *et al*., 2001; Whitham *et al*., 1996). *Rx* has been shown to be active against a range of potexviruses in transgenic *Nicotiana* spp., even when as little as 40% homology exists between the CPs of the viruses concerned (Baurès *et al*., 2008; Candresse *et al*., 2010). As a result of this unusually broad activity range, it has been proposed that *Rx*-based recognition is dependent on conserved structural elements of the viral CP, rather than on a linear amino acid sequence (Baurès *et al*., 2008; Chapman *et al*., 1992; Goulden and Baulcombe, 1993). Transient expression of CP fragments from PVX, *White clover mosaic virus* (WClMV) and *Narcissus mosaic virus* (NMV) has allowed the identification of a 90-amino-acid 'minimal elicitor' region required for *Rx*-based recognition (Baurès *et al*., 2008).

Pepino mosaic virus (PepMV), an emergent potexvirus that presents a major threat to tomato production, also possesses the ability to infect a number of other solanaceous crops. Despite control efforts, tomato-infecting isolates of PepMV have gained a worldwide distribution in just over 10 years (Hanssen *et al*., 2010). A high level of conservation is displayed between all sequenced PepMV isolates in the *Rx* minimal elicitor region of the CP (Candresse *et al*., 2010). *Rx* has been shown to be active against PepMV, providing initial hopes that it may provide a valuable source of resistance in susceptible crop species. However, recent evidence has indicated that *Rx*-based resistance against PepMV in tomato may not be durable. Candresse *et al*. (2010) passaged PepMV through *Rx*-expressing tomato and reported the frequent selection of resistance-breaking isolates. Sequence analysis of the CP of these variants showed the accumulation of a number of point mutations in the *Rx* minimal elicitor region that were proposed to affect *Rx*-mediated recognition (Candresse *et al*., 2010). However, the precise impact of the observed mutations could not be confirmed in the absence of an infectious clone (IC) in which to introduce the suspected point mutations.

Using our recently described ICs of an EU genotype of PepMV (Duff-Farrier *et al*., 2015; GenBank accession; KJ018164), we report the analysis of the impact of two of these candidate point mutations (A-T78, A-T114) in the *Rx* minimal elicitor region. The *Rx*-breaking activity of the resulting PepMV mutants was investigated in three different transgenic *Rx*-expressing hosts: *Nicotiana benthamiana*, *Nicotiana tabacum and Solanum lycopersicum*.

The desired mutations (A-T78 and A-T114) were introduced into the CP region of a wild-type PepMV EU IC, constructed as described previously and contained within a pYES2 vector, pYES2_EU (Duff-Farrier *et al*., 2015). pYES2_EU was used as the template in Phusion PCR (Thermo Scientific, Wilmington, DE, USA) to amplify the PepMV CP region (primers 1 + 2, Table 1), which was cloned into pJET1.2 (Thermo Scientific), forming pJET1.2_CP. This was entered into two site-directed mutagenesis reactions using a GeneArt® Site-Directed Mutagenesis System (Invitrogen, Carlsbad, CA), according to the manufacturer's protocol; mutations A-T78 and A-T114 used primer sets 3 + 4 and 5 + 6, respectively (Table 1). The mutagenized CP regions were excised and entered into a yeast recombination reaction with digested pYES2_EU and a linearized pYES2 backbone, following the protocol of Gietz and Woods (2002). Restriction digestion and sequencing confirmed successful construct generation for both pYES2_EU_A-T78 and pYES2_EU_A-T114.

A Riboprobe® SP6 System (Promega, Madison, WI, USA), in conjunction with a Ribo m7G Cap Analog (Promega), was used to generate infectious transcripts *in vitro* from wild-type, A-T78 and A-T114 *Kpn*I linearized templates (Foster and Turner, 1998; Turner *et al*., 1994, 1999). Each reaction was immediately inoculated onto the surface of two *N. benthamiana* plants at the three-leaf stage. Plants were kept under glasshouse conditions (18 °C with a 16 h/8 h light/dark cycle). Enzyme-linked immunosorbent assay (ELISA) at 21 days post-inoculation (dpi) showed high absorbance values for all constructs, indicating successful establishment of infection in all instances. All ICs displayed systemic phenotypes akin to that of the mild EU IC, characterized by light mosaics (data not shown). For each of the ICs, the CP region was amplified by Phusion PCR (Thermo Scientific) (primers 1 + 2, Table 1) and directly sequenced. Retention of the desired mutations was con-

firmed in progeny of the two mutant ICs; however, secondary mutations in the CP region were also observed (Fig. 1). The progenies of the parental IC and of the A-T78 mutant were found to contain an additional V-A230 mutation, whereas the A-T114 mutant progeny contained an additional E-K236 mutation.

Homogenates from the sequenced primary *N. benthamiana* infections were used to inoculate wild-type and *Rx*-expressing *N. benthamiana*, *S. lycopersicum* (cv. Microtom) and *N. tabacum* (cv. Samsun) (Bendahmane *et al*., 1999; Candresse *et al*., 2010). Triplicate plants of each host type were inoculated with sap representing each IC. Plants were grown as outlined previously. A phenotypic analysis and evaluation of systemic viral accumulation were carried out at 21 dpi. ELISA readily detected the wild-type EU IC in all wild-type hosts, indicating full capacity for systemic movement and accumulation (Fig. 2). The systemic infection phenotypes were characterized by light mosaics in *N. benthamiana* (Fig. 3A), but by asymptomatic infection in *S. lycopersicum* (Fig. 3B) and

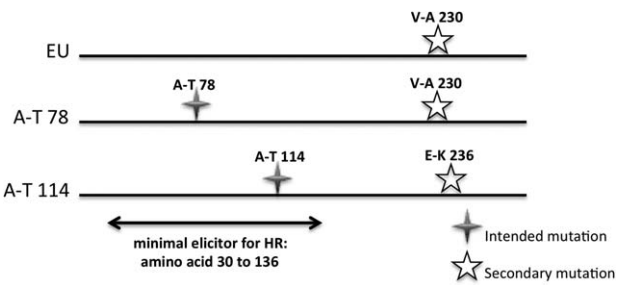

Fig. 1 Consensus sequences of the *Pepino mosaic virus* (PepMV) capsid protein (CP) regions obtained from systemic leaves of *Nicotiana benthamiana* after inoculation with *in vitro*-generated RNA representing each infectious clone (IC): EU, A-T78 and A-T114. Grey crosses indicate intended mutations and white stars indicate secondary mutations.

N. tabacum (data not shown). In contrast with the wild-type plants, background ELISA values were observed and a general absence of symptoms on upper non-inoculated leaves for the *Rx* hosts, indicating an *Rx*-specific inhibition of viral systemic infection (Fig. 2B,C). However, necrotic local lesions were observed on the inoculated leaves of *Rx*-expressing *N. benthamiana* (Fig. 3C, panel A). Necrosis around the site of inoculation was also observed in *S. lycopersicum* (Fig. 3D), but not in *N. tabacum* (data not shown).

RNA was extracted from systemically infected leaves of the wild-type plants using an RNeasy Plant Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. The CP region was amplified as described above and cloned into pJET1.2 (Thermo Scientific). At least two clones were sequenced for each sample. As it is widely known that potexviral CP is the sole elicitor of the *Rx*-based resistance response (Bendahmane *et al*., 1995, 1999; Goulden *et al*., 1993), only the CP region was analysed in this work. In all three hosts, the V-A230 mutation previously observed in the inoculum source was retained in the sequenced progenies (Table 2).

Similar to the wild-type EU IC parent, mutant A-T78 was able to systemically infect all wild-type host species, as indicated by ELISA values comparable with those observed in the wild-type infections (Fig. 2). Again, light mosaics were observed in *N. benthamiana* (Fig. 3A), but asymptomatic infection in both *S. lycopersicum* (Fig. 3B) and *N. tabacum* (data not shown). High ELISA values were observed in the non-inoculated tissues of all *Rx*-expressing *N. benthamiana* and *S. lycopersicum*, indicating a breakdown of *Rx* resistance in these hosts (Fig. 2A,B). Infection phenotypes were characterized by vascular necrosis in the upper parts of the plant in *N. benthamiana* (Fig. 3A,E), and by trailing necrosis over the entire plant in *S. lycopersicum*, with the plant showing a very

Table 2 Mutational composition of clones sequenced from systemic infections generated in both *Rx*-expressing and wild-type (WT) hosts from challenge with both WT and mutant infectious clones (ICs). Intended mutations (italics), secondary mutations and infection phenotypes are indicated.

Host		Genotype/ phenotype	EU WT	A-T78	A-T114
Nicotiana benthamiana	WT (inoculum source)	G	100% V-A230	100% *A-T78*, V-A230	100% *A-T114*, E-K236
		P	Systemic light mosaics	Systemic light mosaics	Systemic light mosaics
	WT	G	100% V-A230	100% *A-T78*, V-A230	100% *A-T114*, V-A230
		P	Systemic light mosaics	–	Systemic light mosaics
	Rx	G	–	66.6% *A-T78*, V-A230 33.3% D-E3, V-A230	–
		P	Local necrosis	Severe local necrosis Systemic vascular necrosis	Severe local necrosis
Nicotiana tabacum	WT	G	100% V-A230	100% *A-T78*, V-A230	100% D-E3, V-A230
		P	Asymptomatic	Asymptomatic	Systemic light mosaics
	Rx	G	–	–	–
		P	–	–	–
Solanum lycopersicum	WT	G	100% V-A230	100% *A-T78*, V-A230	100% *A-T114*, V-A230
		P	Asymptomatic	Asymptomatic	Systemic light mosaics
	Rx	G	–	100% *A-T78*, V-A230	75% *A-T114*, A-D100, V-A230 25% *A-T114*, V-A230
		P	Local necrosis	Severe systemic trailing necrosis	Trailing systemic necrosis

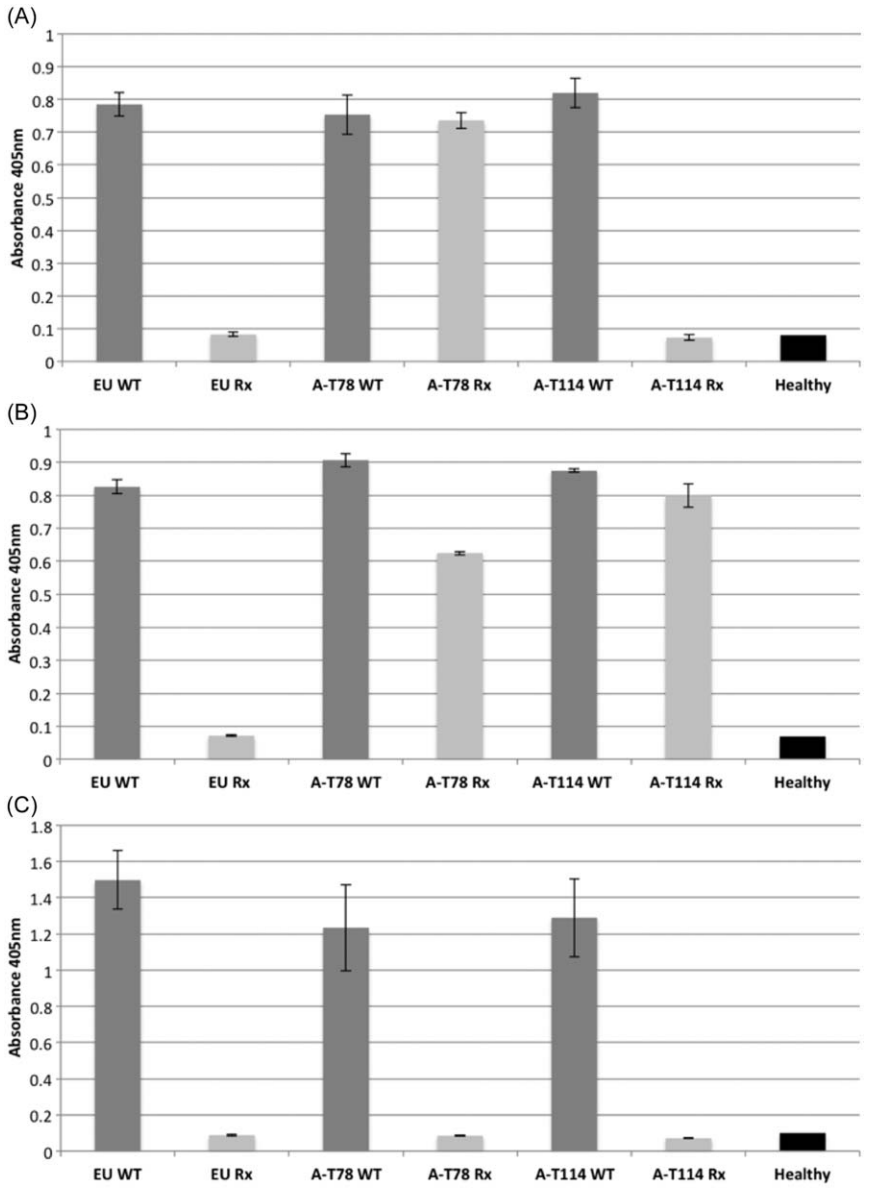

Fig. 2 Double antibody sandwich-enzyme-linked immunosorbent assay (DAS-ELISA) data displaying systemic viral titres at 21 days post-inoculation (dpi) of *Pepino mosaic virus* (PepMV) infectious clones (ICs), A-T78, A-T114 and unmutated EU, in *Rx*-expressing (Rx) and wild-type (WT) solanaceous hosts: (A) *Nicotiana benthamiana*; (B) *Solanum lycopersicum* cv. Microtom; (C) *Nictotiana tabacum* cv. Samsun.

stunted phenotype (Fig. 3B). A phenotype of spreading necrosis was also observed in the inoculated leaves of *N. benthamiana* (Fig. 3C, panel B). In contrast with the situation in *Rx*-expressing *N. benthamiana* and *S. lycopersicum*, no symptoms or systemic viral accumulation could be detected in the *Rx*-expressing *N. tabacum* plants, indicating that mutant A-T78 could not evade the action of *Rx* in this host.

RNA was extracted from systemically infected leaves of both wild-type and *Rx*-expressing plants where infection had established (one plant representing each infection event), and the CP regions of the viral progenies were sequenced as described above; the results are given in Table 2. The introduced A-T78 mutation and the V-A230 secondary mutation previously detected in the inoculum were retained in all progenies sequenced. However, in a third of progeny clones obtained from the systemic leaves of *Rx*-expressing *N. benthamiana*, the A-T78 mutation was lost and, instead, a D-E3 mutation was observed.

Similar to the A-T78 mutant, the A-T114 mutant possessed full systemic accumulation capacity in all wild-type hosts, indicated by positive ELISA values comparable with those of the parental isolate (Fig. 2). The infection phenotypes were also similar to those observed for the wild-type EU IC (Fig. 3A,B). The *Rx* resistance-breaking capability of this mutant was also found to differ between the three tested *Rx*-expressing hosts. Systemic accumulation levels similar to those in the wild-type host were only observed in *S. lycopersicum*, indicating *Rx* breaking in this host (Fig. 2B), and the plants showed a trailing necrosis phenotype (Fig. 3B). However, no symptoms in non-inoculated tissues and no

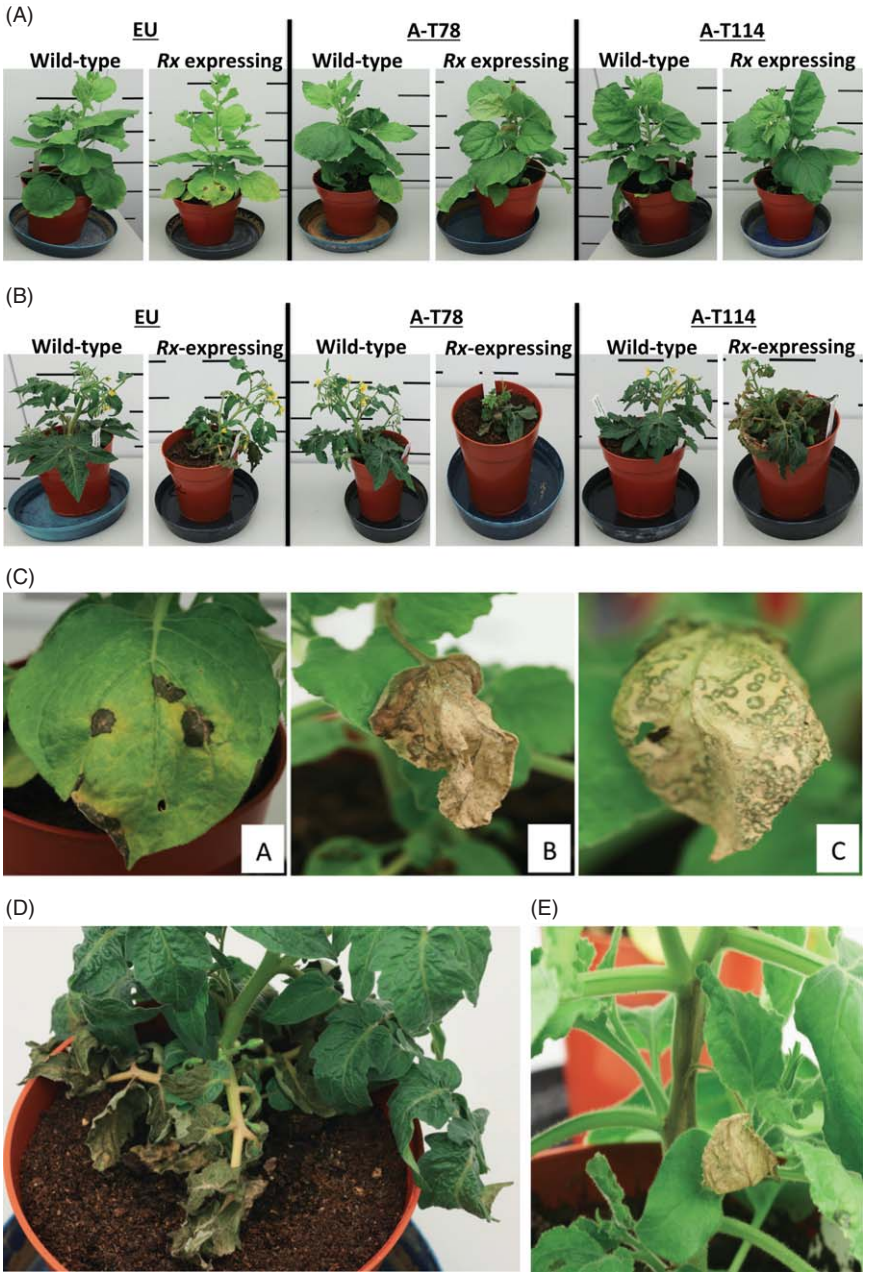

Fig. 3 (A) Representative phenotypes displayed in wild-type and *Rx*-expressing *Nicotiana benthamiana* by the wild-type EU infectious clone (IC) and capsid protein (CP) point mutation ICs A-T78 and A-T114. All ICs presented mild phenotypes in the wild-type hosts. Both EU and A-T114 were asymptomatic in the *Rx* host, whereas A-T78 presented vascular necrosis in the upper parts of the plant. Symptoms were viewed at 21 days post-inoculation (dpi). (B) Representative phenotypes displayed in wild-type and *Rx*-expressing (Rx) *Solanum lycopersicum* by ICs EU, A-T78 and A-T114. All ICs displayed asymptomatic phenotypes in the wild-type hosts. In the *Rx*-expressing hosts, the EU IC caused basal necrosis around the site of inoculation, whereas both A-T78 and A-T114 displayed trailing necrosis over the plant surface. Symptoms were viewed at 21 dpi. (C) Responses observed in the inoculated leaves of *Rx*-expressing *N. benthamiana*, challenged with: A, EU IC; B, mutant A-T78; C, mutant A-T114. Symptoms were viewed at 21 dpi. (D) Close up of basal necrosis around the site of infection in *Rx*-expressing *S. lycopersicum* challenged with the wild-type EU IC. Symptoms were viewed at 21 dpi. (E) Close up of vascular necrosis in *Rx*-expressing *N. benthamiana* challenged with mutant A-T78. Symptoms were viewed at 21 dpi.

systemic accumulation could be observed for this mutant in *N. benthamiana* or *N. tabacum Rx*-expressors (Fig. 2A,C). A local necrotic response was observed in the inoculated leaves of *Rx*-expressing *N. benthamiana* (Fig. 3C, panel C), characterized by circular necrotic lesions within a background of complete leaf necrosis. Sequencing of CP regions was conducted for all progenies as described above; the results are given in Table 2. The E-K236 secondary mutation that had been identified in the inoculum was lost from all progenies. Instead, the same V-A230 mutation present in the inoculum source, progeny of the wild-type parent and progeny of the A-T78 mutant was observed. In tomato, the introduced A-T114 mutation was retained in all progenies irrespective of the *Rx* status of the plants, but was accompanied by an A-D100 secondary mutation in 75% of progeny clones obtained from *Rx*-expressing plants. In wild-type *N. benthamiana*, the A-T114 mutation was retained in all instances. On the contrary, it was absent in all progeny clones obtained from *N. tabacum* and, instead, a secondary D-E3 mutation was observed.

This work describes the analysis of the impact of the introduction of two point mutations on the infection phenotype in wild-type and *Rx*-expressing plants of three host species. These mutations in the *Rx* minimal elicitor region of an *Rx*-sensitive PepMV IC of the EU genotype were selected because they were expected to confer *Rx*-breaking properties (Candresse *et al.*,

2010). The wild-type EU IC possessed full capacity for systemic movement and accumulation in all three wild-type hosts tested but, as expected from previous reports (Baurès *et al*., 2008; Candresse *et al*., 2010), it was efficiently and specifically restricted in all *Rx*-expressing hosts, further confirming that PepMV is recognized by the *Rx*-sensing mechanism. It is interesting to note that localized necrotic responses were observed both at and around the site of inoculation for *N. benthamiana* and *S. lycopersicum*, whereas no such reaction was observed in *N. tabacum*, possibly as a consequence of *Rx* functioning more efficiently in this host. Previous work has shown that *Rx* confers a complete ER phenotype when confronted with a range of different avirulent potexviruses, including PepMV (Baurès *et al*., 2008; Candresse *et al*., 2010). One possibility for this discrepancy is that work carried out by Candresse *et al*. (2010) concerned the CH2 genotype of PepMV, whereas the IC used in the present investigation was of the EU strain.

A secondary mutation, V-A230, was observed in all wild-type EU IC progeny, as well as in almost all progenies derived from the two mutants, the sole exception of which was the first progeny obtained in wild-type *N. benthamiana* for mutant A-T114. This mutation was observed irrespective of the *Rx* status of the host species, suggesting that its highly reproducible accumulation probably reflects the reversion of a detrimental mutation present in the parental IC. In keeping with this interpretation, the alanine at position 230 is highly conserved among PepMV isolates and only absent in three of 82 PepMV CP sequences present in GenBank, all three deriving from the EU IC used in the present experiments. However, the E-K236 mutation observed in the A-T114 inoculum, but lost on further propagation, is probably the result of unselected genetic drift. The same could be true for the D-E3 mutation observed in the progeny of the same mutant on propagation in wild-type tobacco, but this remains to be conclusively demonstrated.

Mutation A-T78 was sufficient to confer *Rx*-breaking properties in both *N. benthamiana* and *S. lycopersicum*, but not in *N. tabacum*, without a need for any additional secondary mutation in the CP. This result is in line with previous work, where the A-T78 mutation was identified alone in the CP region of *Rx* resistance-breaking variants of PepMV in tomato (Candresse *et al*., 2010). In contrast with the A-T78 mutant, A-T114 was only able to overcome *Rx* in *S. lycopersicum* and not in *N. benthamiana* or *N. tabacum*. The role of mutation A-T114 in conferring *Rx*-breaking activity in tomato is less clear-cut than for A-T78, as a secondary A-D100 mutation was also observed in the majority of clones sequenced.

It would appear that mutation A-T114 confers *Rx*-breaking activity in *S. lycopersicum* as it was observed alone in one of the progenies. However, its frequent association with a second compensatory mutation, such as A-D100 reported here or A-V71 previously observed together with A-T114 in spontaneous *Rx*-breaking mutants (Candresse *et al*., 2010), suggests that these secondary mutations may either improve the *Rx*-breaking ability or the fitness of the A-T114 mutant. In all cases, *Rx* breaking in *S. lycopersicum* was accompanied by a spreading necrosis phenotype (Candresse *et al*., 2010; present work), which is *Rx* mediated, as it is not observed in wild-type tomato. The secondary A-D100 mutation was observed in the majority of sequenced clones from *Rx* tomato, yet was absent from all sequences obtained from wild-type tomato, indicating that its compensatory role in *Rx* breaking or in restoring the fitness of the A-T114 mutant is *Rx* dependent. Interestingly, this same A-D100 mutation has been observed in the CP region of two PepMV resistance-breaking variants in *S. lycopersicum* (Candresse *et al*., 2010), alongside Q-R125 in one variant, and with both A-T78 and Q-R125 in another.

The results of this investigation show that *Rx* possesses a high level of recognition in tobacco, as no resistance breaking is observed and systemic movement of the virus is halted. In *N. benthamiana*, *Rx* recognition is intermediate. A-T114 is recognized (local lesions), but not localized, whereas A-T78 is not recognized and displays complete systemic movement capability. In *S. lycopersicum*, recognition is weakest and both mutants, although still recognized, evade localization and overcome resistance. Evidence for intermediate elicitor recognition phenotypes observed in CP–*Rx*-based systems shows that the intensity of the response may vary, clearly based on the strength of protein–protein interactions (Baurès *et al*., 2008; Sturbois *et al*., 2012). Indeed, the findings of this investigation nicely parallel those of Sturbois *et al*. (2012), whereby different tomato mutants were found to possess different interaction phenotypes when confronted with a mutant PVX isolate of intermediate *Rx* elicitor activity. Work by Harris *et al*. (2013) concerning the artificial evolution of *Rx* found that extending the range of *Rx* recognition to include *Poplar mosaic virus* (PopMV) could come with a cost of systemic trailing necrosis. The *Rx* resistance response was demonstrated to consist of separate recognition and activation phases, with PopMV being recognized, but a delayed or incomplete activation of Rx resulting in an inability to suppress viral movement and in a trailing HR phenotype. The necrotic symptoms caused by PepMV mutants in *Rx*-expressing hosts in this work suggest that the mutants are similarly recognized, but not localized, because of host-specific differences in the sensing or downstream signalling in the heterologous hosts studied.

Another point to consider may be host-dependent fitness penalties associated with each mutation. However, fitness penalties associated with the debilitating T-K121 mutation in PVX mutants are seen in non-*Rx* hosts (Goulden *et al*., 1993). On the contrary, the equal levels of accumulation displayed by the various mutants in the wild-type hosts in this work makes this mechanism unlikely.

In conclusion, the results of this investigation support the guard hypothesis of *R*-gene functionality (Dangl and Jones, 2001), which implies the existence of host adaptors that may

contribute to resistance efficiency and durability. Understanding the mechanisms underlying the increased durability of *Rx*-based resistance in the *N. tabacum* host may be integral if the *Rx* gene is to provide a suitable form of resistance against PepMV in tomato. This is a much more complex system than previously thought; the cellular environment in which *Rx* is expressed is integral in its functionality.

ACKNOWLEDGEMENTS

This work was supported by funding from the Lady Emily Smyth Agricultural Research Station (LESARS). We would like to thank The Food and Environment Research Agency (FERA) for use of their facilities and the invaluable work of the glasshouse team in growing the plants used in this investigation. We would also like to thank Bénédicte Sturbois for the kind provision of the *Rx*-expressing and wild-type plant lines used in this study. This work was carried out under Department for Environment, Food and Rural Affairs (DEFRA) license 50973/198463/1.

REFERENCES

Baurès, I., Candresse, T., Leveau, A., Bendahmane, A. and Sturbois, B. (2008) The Rx gene confers resistance to a range of potexviruses in transgenic *Nicotiana* plants. *Mol. Plant–Microbe Interact.* **21**, 1154–1164.

Bendahmane, A., Kohm, 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.

Bendahmane, A., Kanyuka, K. and Baulcombe, D.C. (1999) The Rx gene from potato controls separate virus resistance and cell death responses. *Plant Cell*, **11**, 781–791.

Candresse, T., Marais, A., Faure, C., Dubrana, M.P., Gombert, J. and Bendahmane, A. (2010) Multiple coat protein mutations abolish recognition of Pepino mosaic potexvirus (PepMV) by the potato Rx resistance gene in transgenic tomatoes. *Mol. Plant–Microbe Interact.* **23**, 376–383.

Chapman, S., Hills, G., Watts, J. and Baulcombe, D. (1992) Mutational analysis of the coat protein gene of Potato virus X—effects on virion morphology and viral pathogenicity. *Virology*, **191**, 223–230.

Cockerham, G. (1970) Genetical studies on resistance to Potato viruses X and Y. *Heredity*, **25**, 309–348.

Dangl, J.L. and Jones, J.D.G. (2001) Plant pathogens and integrated defence responses to infection. *Nature*, **411**, 826–833.

Duff-Farrier, C.R.A., Bailey, A.M., Boonham, N. and Foster, G.D. (2015) A pathogenicity determinant maps to the N-terminal coat protein region of the Pepino mosaic virus genome. *Mol. Plant Pathol.* **16**, 308–315.

Farnham, G. and Baulcombe, D.C. (2006) Artificial evolution extends the spectrum of viruses that are targeted by a disease-resistance gene from potato. *Proc. Natl. Acad. Sci. USA*, **103**, 18 828–18 833.

Flor, H.H. (1971) Current status of gene-for-gene concept. *Annu. Rev. Phytopathol.* **9**, 275–296.

Foster, G.D. and Turner, R. (1998) In vitro transcription and translation. In: *Plant Virology Protocols: From Virus Isolation to Transgenic Resistance Methods in Molecular Biology* (Foster, G.D. and Taylor, S.C., eds), pp. 293–299. Totowa, NJ: Humana Press Inc.

Gietz, R.D. and Woods, R.A. (2002) Transformation of yeast by lithium acetate/single-stranded carrier DNA/polyethylene glycol method. *Methods Enzymol.* **350**, 87–96.

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., Cruz, S.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.

Hanssen, I.M., Lapidot, M. and Thomma, B.P.H.J. (2010) Emerging viral diseases of tomato crops. *Mol. Plant–Microbe Interact.* **23**, 539–548.

Harris, C.J., Slootweg, E.J., Goverse, A. and Baulcombe, D.C. (2013) Stepwise artificial evolution of a plant disease resistance gene. *Proc. Natl. Acad. Sci. USA*, **110**, 21 189–21 194.

Jones, J.D.G. and Dangl, J.L. (2006) The plant immune system. *Nature*, **444**, 323–329.

Jones, R.A.C. (1985) Further studies on resistance breaking strains of Potato virus X. *Plant Pathol.* **34**, 182–189.

Moffett, P., Farnham, G., Peart, J. and Baulcombe, D.C. (2002) Interaction between domains of a plant NBS-LRR protein in disease resistance-related cell death. *EMBO J.* **21**, 4511–4519.

Moreira, A., Jones, R.A.C. and Fribourg, C.E. (1980) Properties of a resistance-breaking strain of Potato virus X. *Ann. Appl. Biol.* **95**, 93–103.

Rommens, C.M.T., Salmeron, J.M., Oldroyd, G.E.D. and Staskawicz, B.J. (1995) Intergeneric transfer and functional expression of the tomato disease resistance gene Pto. *Plant Cell*, **7**, 1537–1544.

Song, J., Bradeen, J.M., Naess, S.K., Raasch, J.A., Wielgus, S.M., Haberlach, G.T., Liu, J., Kuang, H., Austin-Phillips, S., Buell, C.R., Helgeson, J.P. and Jiang, J. (2003) Gene RB cloned from *Solanum bulbocastanum* confers broad spectrum resistance to potato late blight. *Proc. Natl. Acad. Sci. USA*, **100**, 9128–9133.

Spassova, M.I., Prins, T.W., Folkertsma, R.T., Klein-lankhorst, M., Goldbach, R.W. and Prins, M. (2001) The tomato gene Sw5 is a member of the coiled coil, nucleotide binding, leucine-rich repeat class of plant resistance genes and confers resistance to TSWV in tobacco. *Mol. Breed.* **220**, 151–161.

Sturbois, B., Dubrana-Ourabah, M.-P., Gombert, J., Lasseur, B., Macquet, A., Faure, C., Bendahmane, A., Baurès, I. and Candresse, T. (2012) Identification and characterization of tomato mutants affected in the Rx-mediated resistance to PVX isolates. *Mol. Plant–Microbe Interact.* **25**, 341–354.

Tozzini, A.C., Ceriani, M.F., Saladrigas, M.V. and Hopp, H.E. (1991) Extreme resistance to infection by Potato virus X in genotypes of wild tuber-bearing solanum species. *Potato Res.* **34**, 317–324.

Turner, R., Bate, N., Twell, D. and Foster, G.D. (1994) Analysis of a translational enhancer upstream from the coat protein open reading frame of Potato virus S. *Arch. Virol.* **134**, 321–333.

Turner, R.L., Glynn, M., Taylor, S.C., Cheung, M.-K., Spurr, C., Twell, D. and Foster, G.D. (1999) Analysis of a translational enhancer present within the 5'-terminal sequence of the genomic RNA of Potato virus S. *Arch. Virol.* **144**, 1451–1461.

Whitham, S., McCormick, S. and Baker, B. (1996) The N gene of tobacco confers resistance to Tobacco mosaic virus in transgenic tomato. *Proc. Natl. Acad. Sci. USA*, **93**, 8776–8781.