Variants of *Peach latent mosaic viroid* inducing peach calico: uneven distribution in infected plants and requirements of the insertion containing the pathogenicity determinant

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Previous characterization of *Peach latent mosaic viroid* (PLMVd) variants from a single peach calico (PC) isolate showed that PC symptoms are induced by variants with a 12–13 nt insertion at a specific position and folding into a hairpin with a U-rich loop. Here, this study was extended to two other PC isolates. PLMVd variants with insertions similar to those reported previously (type 1), predominated in one isolate (PC-P2). The second (PC-P1), in addition to these variants, contained others with insertions in the same position and of the same size, but with the hairpin capped by a GA-rich loop (type 2). When symptomatic and non-symptomatic tissues from both isolates were used to inoculate GF-305 peach seedlings, they reproduced the phenotype of the inoculum source, indicating that variants differing in pathogenicity are unevenly distributed within single plants. Moreover, characterization of the progeny from inoculations with the PC-P1 source showed that variants with insertions of type 1 and 2 were predominant in the symptomatic and non-symptomatic seedlings, respectively, confirming the association between PC and variants with type 1 but not type 2 insertions. Inoculations with dimeric in vitro transcripts from PLMVd variants with type 1, type 2 and with a chimeric insertion showed that the variant with type 2 insertion was latent and established that the U-rich capping loop has a major role in PC, although the adjacent stem may also have some influence. Insertions can be acquired and lost during infection, suggesting that latent variants can evolve into pathogenic variants and vice versa.

INTRODUCTION

Scientific interest in viroids, infectious agents present only in higher plants, has been generated by their unusual properties (Diener, 2001) and by the need to control the losses they cause to crops (Randles, 2003). Viroids, despite having only a small circular RNA genome of 246–401 nt, are able to replicate autonomously through an RNA-based rolling-circle mechanism and to incite specific diseases in certain sensitive hosts (Tabler & Tsagris, 2004; Flores *et al*., 2005a). Because viroids apparently lack protein-coding capacity, they rely very much on host machinery for their replication and movement, and their primary pathogenic effects must result from direct interaction between the genomic or some viroid-derived RNAs and host factors. Therefore, these pathogens offer unique opportunities for studying RNA structure–function relationships and, particularly, for unveiling the mechanisms involved in symptom development elicited by a small non-protein-coding RNA.

Although the molecular mechanisms underlying pathogenesis induced by viroids remain largely unknown, it is generally accepted that they modify host-gene expression, thus interfering with the normal developmental pathways. Until recently, the mature viroid RNA, or some of its replicative intermediates, have been regarded as the primary pathogenic effectors interacting with a host protein or RNA (Diener, 2001). This primary interaction should in principle be diverse for members belonging to the two viroid families, *Pospiviroidae* and *Avsunviroidae*, because they differ in structural, biological and biochemical features (Flores *et al*., 2005b). Indeed, members of the family *Pospiviroidae*, type species *Potato spindle tuber viroid* (Diener, 1972; Gross

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et al., 1978), share conserved motifs including a central conserved region, replicate and accumulate in the nucleus, and do not exhibit RNA self-cleavage mediated by hammerhead ribozymes. In contrast, members of the family Avsunviroidae, type species Avocado sunblotch viroid (Symons, 1981; Hutchins et al., 1986), do not have a central conserved region, replicate and accumulate in the chloroplast, and their replicative intermediates of both polarities self-cleave through hammerhead ribozymes (Flores et al., 2000). In the last few years, however, a new paradigm has emerged proposing that viroid symptoms could result from RNA silencing effects downregulating the expression of certain host genes (Papaefthimiou et al., 2001). Although conclusive evidence demonstrating that RNA silencing is involved in viroid pathogenesis is still lacking (Flores et al., 2005a), this hypothesis is intriguing, as the same mechanism could mediate pathogenesis in both viroid families and even in certain satellite RNAs (Wang et al., 2004).

In addition to the cascade of molecular events leading to symptom expression, a critical step towards understanding viroid pathogenesis is the dissection of viroid genomes to identify the determinants modulating their virulence. Such determinants have been mapped for Potato spindle tuber viroid and other representative members of the family Propiviroidae at small motifs within specific domains of rod-like secondary structure proposed for these RNAs (Gross et al., 1981; Visvader & Symons, 1986; Owens et al., 1996; Reanwarakorn & Semancik, 1998; Schmitz & Riesner, 1998; Qi & Ding, 2003). In the family Avsunviroidae, the pathogenicity determinant of Chrysanthemum chlorotic mottle viroid (CCMVd) (Navarro & Flores, 1997) has been mapped at a tetraloop of its branched secondary structure stabilized by a pseudoknot (De la Peña et al., 1999; De la Peña & Flores, 2002; Gago et al., 2005). Peach latent mosaic viroid (PLMVd), which adopts a similar branched secondary structure (Hernández & Flores, 1992; Ambrós et al., 1998; Pelchat et al., 2000), also stabilized by a pseudoknot (Bussière et al., 2000), is able either to replicate symptomlessly or to induce a broad variety of symptoms that include an extreme chlorosis of leaves, stems and fruits denoted peach calico (PC). Recently, molecular characterization of a PC isolate in combination with reverse genetics experiments has shown that PLMVd variants inducing PC have a size of 348–351 nt, slightly longer than that of typical variants (336–338 nt) from non-symptomatic and mosaic-inducing isolates, due to an insertion of 12–13 nt. This insertion maps at the loop A capping the so-called hammerhead arm (Ambrós et al., 1998), has limited sequence variability, folds itself into a hairpin and appears to emerge sporadically de novo (Malfitano et al., 2003). However, whether the whole 12–13 nt insertion, or a minor portion thereof, is needed for inducing PC remains to be determined, because the 12–13 nt insertion might have to fulfill a structural requirement in order to be preserved. This view is supported by the sequence heterogeneity found in the insertion, which is consistent with the existence of a hairpin with a 4–6 bp stem capped by a 4–5 nt loop (Malfitano et al., 2003).

Here, we have addressed this issue by extending our studies to PLMVd variants from two novel PC isolates. Our results indicated that, while the position, size and folding are structural constrains of the insertions, their sequence can be very different. We showed that natural variants with insertions capped by a GA-rich hairpin loop are latent, whereas the PC phenotype is determined by variants containing insertions with a U-rich hairpin loop, although the adjacent stem appears to have some influence. We also provided data on the segregation within a single plant of variants with different pathogenicity and on the in vivo dynamics of the insertions.

**METHODS**

**Field sources and bioassays.** Two peach trees (Prunus persica Batsch, cultivars Maycrest and Springcrest), naturally infected by PLMVd and showing typical PC symptoms, were selected from two orchards located about 25 km apart from each other in the Apulia region (Southern Italy) and named PC-P1 and PC-P2, respectively. Bioassays were performed by implanting pieces of symptomatic or non-symptomatic bark tissue from each source onto GF-305 peach seedlings. After 6–8 weeks in the greenhouse, the inoculated plants were chilled for 8 weeks at 4°C in darkness and then transferred back to the greenhouse to stimulate growth of new flushes.

**Extraction of nucleic acids and molecular hybridization.** For detection and quantification of nucleic acids, aliquots (5 μl) of 1/5, 1/25 and 1/125 dilutions of nucleic acid preparations extracted from 2 g leaf tissue (Ambrós et al., 1995) were spotted onto positively charged nylon membranes (Roche Diagnostics) and hybridized with a PLMVd-specific digoxigenin-labelled riboprobe. Membranes were washed and incubated with an anti-digoxigenin antibody conjugated to alkaline phosphatase before adding the chemiluminescent substrate CSPD (Roche Diagnostics). For cloning purposes, nucleic acid mini-preparations were obtained with a two-step protocol (Di Serio et al., 2002). Briefly, 25–50 mg leaf tissue was first macerated in an ice-cooled mortar with a pestle and total nucleic acids were extracted with phenol/chloroform and recovered by ethanol precipitation (Dalmay et al., 1993). The nucleic acids were resuspended in 300 μl RNase-free water and treated with a modified silica-gel capture system (Foissac et al., 2001). This procedure reduced the plant material needed for PLMVd cloning to less than 1 cm² fresh leaf tissue.

**Progeny analysis by RT-PCR amplification, cloning and sequencing.** First-strand PLMVd cDNAs were synthesized from aliquots (5 μl) of the nucleic acid mini-preparations with avian myeloblastosis virus reverse transcriptase (Promega) and primer RF-43 (5’-CTGGATCACACCCTCCCGAGACAGACCGTC-3’), complementary to positions 208–178 of the PLMVd reference variant (Hernández & Flores, 1992; Ambrós et al., 1998) and then PCR amplified with Pfu DNA polymerase (Stratagene) and primers RF-43 and RF-44 (5’-TGATGTACGACCGTACGGGAGC-3’), identical to positions 199–225 of the PLMVd reference variant. The resulting products were separated by electrophoresis in 1·2% agarose gels and DNA of the expected size was eluted and cloned into plasmid pUC19 (New England Bio-Labs) digested at the Smal site. Progeny variants recovered from GF-305 seedlings inoculated with the in vitro transcripts corresponding to PC-P1.142 were cloned using the same approach but replacing primers RF-43 and RF-44 with primers PLMV-59 (5’-CTTACCTCATAGGGTCTTACCCG-3’) and PLMV-60 (5’-GTGGGACTTTTCCTCTGGAACC-3’), complementary and identical to positions 128–153 and
154–179 of the reference variant (Hernández & Flores, 1992; Ambros et al., 1998), respectively. Inserts were sequenced automatically with an ABI PRISM DNA apparatus (Perkin-Elmer).

**Construction of plasmids containing head-to-tail PLMVd cDNA dimeric inserts.** Plasmids pGdP1.142 and pGdP1.159, with head-to-tail PLMVd cDNA dimeric inserts, were generated from the recombinant plasmids containing the respective monomeric inserts, which were PCR amplified with Pfu DNA polymerase and the phosphorylated primers FPLMV-57 (5′-CAGCCACCCTCCGGAACCACGAAACCG-3′) and FPLMV-58 (5′-ATCCAGGTACCCGGTATAAGAAC-3′), complementary and identical to positions 202–180 and 203–224 of the reference PLMVd variant (Hernández & Flores, 1992; Ambros et al., 1998), respectively. Following ligation with T4 DNA ligase (Roche Diagnostics), the oligomeric DNA fragments were separated by electrophoresis in 1·2 % agarose gels and those with the expected dimeric size were eluted and cloned into the plasmid pGEM-T Easy (Promega). The recombinant plasmid pSdP1.148, containing the head-to-tail dimeric PLMVd cDNA insert corresponding to variant PC-P1.148, was obtained by using a similar approach and cloned into the plasmid pSP719 (Roche Diagnostics). The resulting plasmids were sequenced in both directions to confirm that no artefactual mutation had been introduced.

**Site-directed mutagenesis.** A PCR-based method was followed to generate the recombinant variant PC-P1.142-UUUU, in which the GAAG loop capping the type 2 inserted hairpin of the natural PC-P1.142 variant was replaced by the UUUU loop (see Fig. 5). Briefly, plasmid pGdP1.142 served as template for PCR amplification with Pfu DNA polymerase and the phosphorylated primers FPLMV-65 (5′-GAACCGAAGTTTGCCTCTATCTC-3′) and FPLMV-66 (5′-ATTGTTGTACCCATAGTTGCGCATCTC-3′), complementary and identical, respectively, to positions 343–318 and nt 344–22 of the PC-P1.142 variant in the multiple alignment (Fig. 1) except in the four contiguous positions 345–348 (underlined) that were mutated to generate and amplify the PC-P1.142-UUUU monomeric PLMVd variant. The corresponding head-to-tail dimeric PLMVd cDNAs were obtained as described in the previous section and cloned into the pGEM-T Easy vector.

**In vitro transcription and RNA inoculation.** Recombinant plasmids containing head-to-tail PLMVd cDNA dimeric inserts were linearized with appropriate restriction enzymes and transcribed with T7 or SP6 RNA polymerase. Transcription products were analysed by electrophoresis in 5 % polyacrylamide gels containing 1 x TBE buffer, 8 M urea and 40 % formamide, and slash-inoculated into GF-305 peach seedlings that were treated as indicated above.

**Sequence analysis and RNA secondary structure prediction.** Multiple sequence alignments were generated by using the CLUSTAL W program (Thompson et al., 1994) with minor manual adjustments. Secondary structures of lowest free energy were calculated with the circular version of the Mfold program (Zuker et al., 1999).

**RESULTS AND DISCUSSION**

**Two new PLMVd isolates inducing PC contain variants with a 12–14 nt insertion**

Groups of nearby peach trees of cultivars Maycrest and Springcrest infected with PLMVd and showing PC were identified in 2001 in two commercial orchards of Apulia (Southern Italy), located far away from each other and from the source of the PC isolate characterized previously in Campania (PC-C) (Malfitano et al., 2003). One representative symptomatic tree from each orchard was chosen and referred to for further analysis as PC-P1 (cv. Maycrest) and PC-P2 (cv. Springcrest). Nucleic acids from a small amount of symptomatic leaf tissue from each isolate were used to generate, via RT-PCR with PLMVd-specific primers, full-length PLMVd cDNAs that were cloned and sequenced. Fig. 1 presents the multiple alignment of the resulting sequence variants. Of the seven variants cloned from isolate PC-P2 (Fig. 1, white background), five were 348–350 nt and resembled the PC-inducing variants reported previously (Malfitano et al., 2003): they showed the characteristic 12 nt insertion (Fig. 1, positions 340–353), which adopts a hairpin conformation and is located in the loop A of the proposed branched secondary structure (Fig. 2a). The hairpin insertion of variants PC-P2.36, PC-P2.46 and PC-P2.43, capped by a UUUU loop, was the same as that found in the PC reference variant (PC-C40) (Malfitano et al., 2003) (Figs 1 and 2), whereas the hairpin insertion of variant PC-P2.48 was capped by a UUUA loop and that of PC-P2.44 showed point mutations disrupting the base pair between the first and last positions (Fig. 2b, box 2). The remaining two PC-P2 variants (PC-P2.35 and PC-P2.42) had a size of 337–338 nt, similar to that of the PLMVd reference variant (Hernández & Flores, 1992) (Fig. 1) and other typical variants (lacking the insertion at loop A) from mosaic-inducing and latent PLMVd isolates (Ambros et al., 1998; Pelchat et al., 2000).

The structure of the PLMVd population characterized in the PC-P1 isolate was different. Although the 12 sequenced variants had a uniform size of 349–350 nt due to the 12–14 nt hairpin inserted in loop A, two groups could be established according to the primary structure of this hairpin (Fig. 1, positions 340–353). The first, formed by eight variants (Fig. 1, dark grey background), showed hairpins similar to that of the reference PC-C40 variant: the capping tetraloop was UUUU (Fig. 2b, box 3), with the nucleotide changes being restricted to the stem of 3 and 4 bp (or of 6 bp if nucleotides outside the insertion are considered) in six and two of the variants, respectively (Fig. 2, box 3). The A→G substitution at position 3 of the inserted hairpin in PC-P1.152 and PC-P1.139 variants did not alter the stem because it converted a canonical into a wobble base pair (P1.152) or was accompanied by a U→C co-variation at position 10 (P1.139) (Fig. 2, box 3). The insertions of this group were called type 1, to distinguish them from the type 2 insertions characteristic of the second group of PC-P1 variants (Fig. 1, light grey background). The four insertions of type 2 were 14 nt long and folded into a hairpin showing extensive changes with respect to that of the PC-C40 variant and other variants characterized so far (Malfitano et al., 2003; this study): the capping tetraloop was GAAG (or a minor modification thereof, GAAA, in variant P1.135) flanked by a stem of 5 bp. The A→G substitution at the penultimate position of the inserted hairpin in PC-P1.150 did not disrupt the stem because it converted a canonical into a wobble base pair, whereas the substitution A→U in the same position of variant PC-P1.146 either reduced the stem to 3 bp or maintained its size if a non-canonical U→U pair could be formed (Fig. 2b, box 4).
The multiple alignment (Fig. 1) also revealed polymorphic positions spread throughout the molecule that, excluding those of the insertion (Fig. 1, positions 340–353) and those at positions 134 (variant PC-P1.145) and 163 (variant PC-P2.46), have been reported previously (Hernández & Flores, 1992; Ambros et al., 1998; Pelchat et al., 2000; Malfitano et al., 2003) and the reference PLMVd variant (PL-ref) (Hernández & Flores, 1992), with and without the hairpin insertion, respectively, are boxed with broken lines and shown on the top for comparative purposes. Nucleotide identity and gaps with respect to the PC-C40 variant are indicated by dots and dashes, respectively. Asterisks between positions 340 and 353 denote the absence in some variants of the 12–14 nt insertion and dark and light grey backgrounds discriminate variants with insertions of type 1 and 2, respectively. Flags delimit regions involved in the formation of plus and minus hammerhead structures, with their self-cleavage sites marked by arrows and nucleotides conserved in most natural hammerhead structures within boxes; solid and open symbols refer to plus and minus polarity, respectively. Numbers on top indicate nucleotide positions in the multiple alignment and numbers at the end the full-length of each variant.

**Fig. 1.** Multiple sequence alignment of PLMVd cDNA variants from isolates PC-P1 (grey background) and PC-P2 (white background). The reference PC variant (PC-C40) (Malfitano et al., 2003) and the reference PLMVd variant (PL-ref) (Hernández & Flores, 1992), with and without the hairpin insertion, respectively, are boxed with broken lines and shown on the top for comparative purposes. Nucleotide identity and gaps with respect to the PC-C40 variant are indicated by dots and dashes, respectively. Asterisks between positions 340 and 353 denote the absence in some variants of the 12–14 nt insertion and dark and light grey backgrounds discriminate variants with insertions of type 1 and 2, respectively. Flags delimit regions involved in the formation of plus and minus hammerhead structures, with their self-cleavage sites marked by arrows and nucleotides conserved in most natural hammerhead structures within boxes; solid and open symbols refer to plus and minus polarity, respectively. Numbers on top indicate nucleotide positions in the multiple alignment and numbers at the end the full-length of each variant.
The sequence heterogeneity found in variants from isolates PC-P1 and PC-P2 did not alter significantly either the branched secondary structure of lowest free energy (Fig. 2a) or the hammerhead structures, which are conserved features of all PLMVd variants. All of the characterized variants of these two isolates were different from one another and from those of the original PC-C isolate, confirming previous data on the high sequence variability of PLMVd populations (Hernández & Flores, 1992; Ambrós et al., 1998, 1999; Pelchat et al., 2000; Malfitano et al., 2003).

Although the concurrent presence of PLMVd pathogenic and latent variants within the same plant has been reported (Malfitano et al., 2003), no attempt was made in that study to characterize separately those present in symptomatic tissues, which should be more directly related to disease induction. Analysing RNA preparations from very small symptomatic leaf areas, we found here that complex populations of variants not containing or containing different insertions co-existed in PC-expressing tissues and that PC was strictly correlated with the presence of variants with an inserted hairpin capped by a U-rich loop. However, the simultaneous identification in the same population of variants containing type 1 insertions with a hairpin stem reduced to 3 bp and, particularly, of variants with a hairpin capped by a GA-rich tetraloop (type 2 insertions), was unexpected and indicated that PLMVd RNA can tolerate different insertions in loop A as long as the hairpin folding is preserved, and left open the possibility that PC symptoms could be elicited by more than one type of insertion.

**PLMVd variants with different insertions are unevenly distributed within PC-symptomatic trees**

PC symptoms can be reproduced easily in the greenhouse by grafting symptomatic bark tissue from field trees onto GF-305 peach seedlings (Malfitano et al., 2003). However, symptoms did not develop when GF-305 plants were inoculated with non-symptomatic material from the same
tree (A. Ragozzino & F. Di Serio, unpublished data). These preliminary observations have now been confirmed and extended based on the behaviour of the two new PC-inducing PLMVd isolates. Using the same inoculation scheme with the isolate PC-P2, which has a variant composition very similar to that of the reference PC isolate (PC-C), we observed that PC symptoms in GF-305 paralleled those of the source tree. Because dot-blot hybridizations showed the presence of PLMVd in all of the inoculated GF-305 seedlings (data not shown), these results indicated that variants differing in pathogenicity accumulate separately in the symptomatic and non-symptomatic tissues of the source tree.

We then reasoned that application of the same approach to isolate PC-P1 could lead to the segregation on individual GF-305 plants of PLMVd populations with distinct biological properties and that characterization of the resulting viroid progenies could provide information about the pathogenicity of variants with type 2 insertions identified in this isolate. To this end, two groups of six GF-305 seedlings were inoculated with symptomatic and non-symptomatic bark tissue from isolate PC-P1. As expected, dot-blot hybridization detected PLMVd in all of the seedlings 6 months post-inoculation (data not shown), and the correlation between PC symptoms (or lack thereof) in the source and in the inoculated plants was confirmed again (Fig. 3 and data not shown). Molecular characterization of viroid progeny from one symptomatic and one non-symptomatic GF-305 seedling showed that both PLMVd populations were composed of variants very similar to those found in the parental PC-P1 source (Fig. 1). Interestingly, although variants with insertions of type 1 or 2 and those with no insertion were recovered from both seedlings, their relative abundance differed depending on the inoculum source (Fig. 3). Variants containing insertions of type 1 were predominant (7/10) in the symptomatic GF-305 seedling (Fig. 3c, left), whereas in the non-symptomatic GF-305 seedling those with type 2 insertions (5/9) and without insertions (3/9) prevailed (Fig. 3c, right).

Collectively, these data support a close association between PC symptoms and PLMVd populations mostly composed

![Fig. 3](image-url). Scheme showing the uneven distribution of symptoms and PLMVd variants with different pathogenic properties in the original source of PC-P1 isolate. (a) Symptoms (white leaves and stems) were restricted to a few branches in the source tree. (b) Graft transmission to GF-305 seedlings led to symptoms that paralleled those of the bark tissues used as inoculum. (c) Hairpin insertions found in the progeny from one representative symptomatic (left) and one non-symptomatic (right) graft-inoculated GF-305, with their relative frequency (indicated by boxed fractions) and sequence variability. The specific variants containing the depicted insertions are listed at the bottom of each box. Variant P1.171 is identical to variant P1.172 (accession no. DQ222066); variants P1.154, P1.166, P1.178 and P1.179 are identical to variant P1.137 (accession no. DQ222044); variants P1.155, P1.156, P1.160 and P1.174 are identical to variant P1.142 (accession no. DQ222047); variants P1.163 and P1.173 are identical to variant P1.139 (accession no. DQ222046). Other details are as in the legend to Fig. 2.
of variants containing insertions of type 1. In line with this view, at least two of the variants recovered from the symptomatic GF-305 seedling (PC-P1.169 and PC-P1.177) could be implicated directly in eliciting the PC disease because their inserted hairpins were identical to that found in the PC-inducing variant PC-C28 characterized previously (Malfitano et al., 2003). On the other hand, the prevalence of variants with insertions of type 2 in the non-symptomatic GF-305 seedling strongly suggested that this structural element is not involved in PC. However, we could not conclude at this stage whether variants containing insertions of type 1 with a stem of only 3 bp were also involved in PC, because these variants were recovered from symptomatic GF-305 tissue together with others with inserted hairpins typical of PC-inducing variants. An additional outcome of these experiments is that PLMVd variants with different structural and biological features are unevenly distributed within a single infected tree. Uneven distribution of variants within a single plant has been reported previously for other members of both viroid families (Semanick & Szychowski, 1994; Skorić et al., 2001), although a direct relationship with the distinct host responses induced by these variants could not be established.

**Role of PLMVd variants containing different insertions in inducing PC**

To gain further insight into the pathogenicity of variants from isolate PC-P1, in vitro-synthesized transcripts from plasmids containing head-to-tail dimeric full-length cDNAs corresponding to variants PC-P1.148, PC-P1.142 and PC-P1.159 (representatives of those with insertions of type 1, type 2 and with no insertion, respectively), were slash-inoculated into blocks of 15 GF-305 peach seedlings. These plants were observed for symptom expression and, to establish a relationship between the structural features of the infecting variants and their pathogenicity, the progeny of the inoculated plants were cloned and sequenced. GF-305 seedlings inoculated with full-length RNA transcripts corresponding to the PC-inducing reference variant PC-C40 served as controls. Dot-blot hybridizations performed 6 months after inoculation showed that all transcripts were infectious (around 80% of the inoculated plants became infected) and led to similar PLMVd accumulation levels in vivo (data not shown). In agreement with previous results (Malfitano et al., 2003), the PC-C40 variant induced PC symptoms in all 12 infected plants and generated variant progeny containing the parental hairpin insertion (Fig. 2b) or a minor modification thereof (a compensatory G→A substitution in the third position not disrupting the hairpin stem) (Fig. 4a), identical to that found in the PC-inducing variant PC-C28 (Malfitano et al., 2003).

Variant PC-P1.148, containing an insertion of type 1 with a 3 bp stem capped by a UUUU loop, also induced PC symptoms but only in four of the 12 infected GF-305 seedlings (Fig. 4c), suggesting that the sequence or structure of the inserted hairpin could influence symptom expression. Sequencing of eight PLMVd variants from one symptomatic and one non-symptomatic (but infected) GF-305 seedling inoculated with this variant showed that the parental type 1 insertion was maintained, with minor variability, in the progenies of both plants (Fig. 4c). This excluded the possibility that lack of PC symptoms in the GF-305 seedling analysed could result from loss of the insertion in the progeny. A more detailed analysis showed that single point mutations in the insertion extended the hairpin stem to 4 bp in three of the eight variants recovered from the symptomatic GF-305 seedling (Fig. 4c, left). Furthermore, in two of these variants (PC-P1.148.87 and PC-P1.148.01), the A→C substitution in the last position of the insertion generated a hairpin identical to that of the PC-inducing variant PC-C28 (Malfitano et al., 2003), suggesting the involvement of at least these variants in eliciting the PC symptoms observed. Similar insertions leading to a more stable hairpin were not found in the progeny from the non-symptomatic GF-305 seedling, which contained the parental insertion or a hairpin with a stem of only 2 bp as a consequence of a point mutation at position 11 of the insertion (Fig. 4c, right).

Overall, these data support the hypothesis that a stem of at least 4 bp in the inserted hairpin may contribute to the PC phenotype.

Variant PC-P1.159, without insertion, was non-symptomatic and its progeny was composed mostly of variants without insertion, although one variant (PC-P1.159.91) presented an insertion of type 1 (Fig. 4b), thus confirming a previous observation indicating that this structural element can be acquired de novo (Malfitano et al., 2003). Variant PC-P1.142 also did not induce any visible symptoms in the 13 infected seedlings (Fig. 4d). In the progeny, one variant conserved the parental type 2 hairpin insertion, but most (5/9) showed modifications in the capping GA-rich loop or in the stem, which was preserved because of several co-variations (Fig. 4d). Interestingly, three variants of this progeny had no insertion, showing that this structural element can also be lost during infection. These data demonstrate that type 2 insertions do not contain the pathogenic determinant for PC or for other leaf symptoms. To check the plant-to-plant reproducibility, the progenies of three pairs of GF-305 seedlings inoculated with variants PC-P1.159, PC-P1.142 and the reference PC variant PC-C40 were compared: in all three cases the variant distribution within each pair was similar (data not shown).

In concert, these bioassays, showing that only variants with inserted hairpins capped by a UUUU loop (PC-C40 and PC-P1.148) were able to incite PC symptoms, point to a major role of such a loop in PC pathogenesis and exclude the possibility that expression of this syndrome could result from a specific combination of variants. The finding that a natural variant with a type 2 insertion capped by a GAAG loop (PC-P1.142) is latent supports this hypothesis. Moreover, characterization of P1.148 progeny from symptomatic and non-symptomatic GF-305 seedlings suggests that not only the capping loop, but also the hairpin stem may play a role in PC. It is possible that the lower efficiency in eliciting
PC of variants containing type 1 insertions with a hairpin stem of 3 bp may be due to the need to incorporate spontaneous mutations enlarging the hairpin stem, which should subsequently accumulate to a certain level in the population before symptom appearance. Because all tested transcripts were infectious and their insertions were preserved in the progeny, we could exclude the possibility that variants with insertions of type 2, or type 1 with a hairpin stem of 3 bp, were cloning artefacts. This conclusion was corroborated by the identification of similar insertions in different cDNA clones from the original source of isolate PC-P1 and from a GF-305 seedling inoculated with symptomatic tissue from this isolate.

**A UUUU loop capping the inserted hairpin is insufficient to induce PC**

The finding of PLMVd RNAs with two insertion types, adopting a similar secondary structure but with different sequence and pathogenic properties, provided the opportunity for the first dissection of the PC determinant to explore the relative contribution of the loop and the stem of type 1 insertions. To establish whether the presence of a UUUU loop capping the hairpin insertion was sufficient to elicit PC, the GAAG loop capping the type 2 insertion of the latent PC-P1.142 variant was substituted with a UUUU loop by site-directed mutagenesis. Dot-blot hybridization showed that the in vitro full-length RNA transcript corresponding to the recombinant variant PC-P1.142-UUUU was highly infectious when inoculated onto GF-305 seedlings, with the resulting progeny accumulating at a level similar to that of the parental variant PC-P1.142 (data not shown). Moreover, the recombinant insertion was stable because most of the 12 progeny variants that were sequenced maintained it or presented point mutations preserving a U-rich tetraloop (Fig. 5). In four variants, however, the tetraloop was substituted by a pentaloop or a hexaloop with high sequence variability. None of the 30 infected seedlings displayed PC symptoms, showing that changing the GAAG loop was insufficient to induce PC.

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**Fig. 4. Symptom expression and hairpin insertions found in the progeny of GF-305 seedlings inoculated with in vitro transcripts from plasmids containing head-to-tail dimeric full-length cDNAs of variants PC-C40 (a), P1.159 (b), P1.148 (c) and P1.142 (d). Each box refers to the results of inoculation with the variant indicated on top. The fraction of symptomatic to infected plants, multiple alignments of the insertions (left: bold letters and dots denote nucleotide mutations and identities with respect to the parental variant, respectively, grey background delimits the nucleotides forming the hairpin loop and asterisks indicate the absence of insertion) and their secondary structures and sequence variability (right: for symbols see legend to Fig. 2) are presented. In the case of variant P1.148, the progeny from both a symptomatic and a non-symptomatic (but infected) GF-305 seedlings are reported. Variants P1.148.32, P1.148.59 and P1.148.45 are identical to variants P1.148.04 (accession no. DQ222086), P1.148.58 (accession no. DQ222095) and P1.148.07 (accession no. DQ222093), respectively; variants P1.142.08 and P1.142.10 are identical to variant P1.159 (accession no. DQ222062).**
Fig. 5. Symptom expression and hairpin insertions found in the progeny of GF-305 seedlings inoculated with in vitro transcripts from a plasmid containing a head-to-tail dimeric full-length cDNA of the P1.142-UUUU variant, derived from P1.142 by site-directed mutagenesis in which the tetraloop GAAG of the inserted hairpin was substituted by UUUU. The fraction of symptomatic to infected plants, multiple alignment of the insertions (left: bold nucleotides and dots denote mutations and identities with respect to the parental variant, respectively, and grey background delimits the nucleotides forming the hairpin loop) and their secondary structures and sequence variability (right: arrow indicates nucleotide insertion; for other symbols, see legend to Fig. 2) are presented.

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to a UUUU loop in the inserted hairpin was insufficient to transform a latent into a PC-inducing variant. Therefore, other structural features, presumably located in the stem of the inserted hairpin, must be involved in the pathogenic properties of this structural element.

Throughout this work, the dot-blot hybridizations performed to test the infectivity of the different PLMVd transcripts produced signals of comparable intensity, thus excluding the possibility that the phenotypes incited could be the consequence of distinct accumulation levels of the resulting progenies. Therefore, it seems more likely that PLMVd variants containing the PC-inducing insertions are competent to interfere specifically with the normal function of a host factor(s). Similar results have been observed with CChMVd pathogenic and latent variants, which provide an intriguing parallel with PLMVd: the major pathogenic determinant of CChMVd maps at a U-rich tetraloop (UUUC), which in latent variants is replaced by a GAAA tetraloop (De la Peña et al., 1999; De la Peña & Flores, 2002). Further molecular dissection of the PLMVd determinant inducing PC, taking into account the results reported here, should help to unravel the role of its primary and secondary structures.

Finally, our results also prompt further considerations on the origin and the evolution of the PLMVd variants with inserted hairpins. A previous study showed that a PLMVd variant from which the PC-inducing insertion had been removed by site-directed mutagenesis was able to recover it during infection, supporting the de novo emergence of this structural domain (Malfitano et al., 2003). Here, we have taken this observation one step further by showing that the inserted hairpins are dynamic elements that can be acquired and lost by natural variants, as illustrated by the PC-P1.159 and PC-P1.142 progenies, respectively (Fig. 4b and d). These findings also suggest that insertions of type 1 and 2 identified in the PLMVd originate independently through mechanisms that remain unknown. The de novo emergence of PC-inducing PLMVd variants is consistent with the characteristic sudden appearance of the PC syndrome in peach trees that had grown previously without symptoms and points out the need to include PLMVd in certification schemes for peach propagative material.


