**Viability and genetic stability of potato spindle tuber viroid mutants with indels in specific loops of the rod-like secondary structure**

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http://dx.doi.org/10.1016/j.virusres.2017.07.024

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**ABSTRACT**

Maintenance of the rod-like structure of potato spindle tuber viroid (PSTVd), which contains over 20 loops and bulges between double-stranded helices, is important for viroid biology. To study tolerance to modifications of the stem-loop structures and PSTVd capacity for mutation repair, we have created 6 mutants carrying 3-4 nucleotides deletions or insertions at three unique restriction sites, *Eag*I, *Sty*I and *Ava*II. Differences in the infectivity of these *in vitro* generated PSTVd mutants can result from where the mutations map, as well as from the extent to which the secondary structure of the molecule is affected. Deletion or insertion of 4 nucleotides at the *Eag*I and *Sty*I sites led to loss of infectivity. However, mutants with deletion (PSTVd-Ava-del) or insertion (PSTVd-Ava-in) of 3 nucleotides (221GAC223), at the *Ava*II site (loop 20) were viable but not genetically stable. In all analyzed plants, reversion to the wild type PSTVd-S23 sequence was observed for the PSTVd-Ava-in mutant a few weeks after agroinfiltration. Analysis of PSTVd-Ava-del progeny allowed the identification of 10 new sequence variants carrying various modifications, some of them having retained the original three nucleotide deletion at the *Ava*II site. Interestingly, other variants gained three nucleotides in the deletion site but did not revert to the original wild type sequence. The genetic stability of the progeny PSTVd-Ava-del sequence variants was evaluated in tomato leaves (early infection) and in both leaves and roots (late infection), respectively.

**Keywords:** agroinfiltration; genetic stability; indel mutants; infectivity; viroid

**1. Introduction**

Viroids are single-stranded, covalently-closed, circular, non-encapsidated RNAs of around 250-400 nt. Despite their extremely simple structure, viroids are able to infect many industrial and ornamental plants and induce in some of them disease symptoms such as stunting, leaf chlorosis and necrosis, or misshapen fruits and tubers (Flores et al., 2015; Palukaitis, 2014; Tsagris et al., 2008). Viroids are classified into two families, *Avsunviroidae* and *Pospiviroidae* (Di Serio et al., 2014).Potato spindle tuber viroid (PSTVd) is the best-known representative of the family *Pospiviroidae*. Like other viroids, PSTVd does not encode any protein and, therefore structural elements of the viroid RNA must efficiently interact with host factors to elicit efficient replication, intra/intercellular and long distance trafficking. From this point of view, the structure (secondary, tertiary) of the viroid molecule and its preservation are crucial. PSTVd RNA is known to fold, *in vitro* and *in vivo*, into a thermodynamically favorable rod-like conformation with 27 loops separated by short helices (Gross et al., 1978; López-Carrasco and Flores, 2016; Zhong et al., 2008). Many mutagenesis studies have indicated the importance of the preservation of this secondary structure (Hammond, 1994; Hammond and Owens, 1987; Owens et al., 1991; Qu et al., 1993), a result also supported by the duplications observed in natural variants of coconut cadang-cadang viroid (Haseloff et al., 1982) and citrus exocortis viroid(Fadda et al., 2003; Semancik et al., 1994) and by the complementary deletion converting a 350 nt non-infectious PSTVd mutant into a 341 nt infectious one (Wassenegger et al., 1994).

The rod-like structure of PSTVd and other members of the genus *Pospiviroid* has been divided into five structural domains named the central (C), pathogenic (P), variable (V), terminal right (TR) and terminal left (TL) (Fig. 1a), which are connected with particular steps of the viroid life cycle (Keese and Symons, 1985). For example the C and TL domains are involved in replication (Bojic et al., 2012; Kolonko et al., 2006; Zhong et al., 2008), while structural elements of both the V and TR domains are connected with trafficking (Qi et al., 2004; Zhong et al., 2007). Viroid invasion of neighboring cells and systemic trafficking is regulated by particular specific loop/bulges in the rod-like PSTVd secondary structure: i) a bipartite RNA motif responsible for unidirectional movement from the bundle sheath to mesophyll cells (Qi et al., 2004); ii) loop 7 (U43/C318), which enables movement from non-vascular to phloem tissue (Zhong et al., 2007); iii) loop 6 (36CGA38/323GAC325 flanked by cis Watson-Crick G/C and G/U wobble base pairs) responsible for trafficking from palisade to spongy mesophyll (Takeda et al., 2011). It is also known that PSTVd transport requires formation of specific RNA-protein complexes. Such complexes have been detected between PSTVd RNA (RY motifs in the TR domain) and Virp1 (Gozmanova et al., 2003; Kalantidis et al., 2007) as well as between hop stunt viroid (HSVd, a member of the genus *Hostuviroid*) and phloem lectin PP2 (Gómez and Pallás, 2001; Owens et al., 2001).

A genome-wide mutational analysis (Zhong et al., 2008) has identified loops in PSTVd structure that are important or essential for viroid replication in single cells or systemic trafficking. To study the degree of indels tolerance within some of the loops in the PSTVd rod-like structure, we have constructed six mutants in three unique restriction sites, *Eag*I, *Sty*I and *Ava*II. In what follows, we evaluated their viability, virulence, genetic stability and have tracked the fate of their progeny in leaves and in roots of infected tomato plants.

**2. Materials and methods**

*2.1. Construction of PSTVd mutants*

For this purpose, a recombinant pUC9 plasmid carrying an infectious monomeric full-length cDNA copy of the PSTVd-S23 (Góra et al., 1994) was used. The viroid cDNA was cut with one of three selected endonucleases: *Eag*I, *Ava*II or *Sty*I, treated with Klenow DNA polymerase (New England BioLabs) to fill in the overhangs or with mung bean nuclease (New England BioLabs) to remove the sticky ends and re-ligated with T4 DNA ligase (Promega). All construct were verified by Sanger sequencing. Following digestion with *Bam*HI, the modified PSTVd-S23 inserts were cloned into the pGreen binary vector (Hellens et al., 2000) with a modified multiple cloning site (Więsyk et al., 2011). The obtained recombinant pGreen plasmids were used to transform the *Agrobacterium tumefaciens* [C58C1] cells carrying the virulence helper plasmid pCH32.

*2.2. Plant inoculation*

Tomato seedlings (*Solanum lycopersicum* cv. ‘Rutgers’) at the two-leaves stage were inoculated by agroinfiltration as described previously (Więsyk et al., 2011) and maintained in a greenhouse at temperature 28-30ºC for 16 h (day) and at 25ºC for 8 h (night). Disease symptoms were monitored weekly. Plant samples were collected from expanded leaves and from roots, and PSTVd infection was assayed by dot-blot hybridization with a PSTVd-specific, digoxigenin-labeled RNA probe (DIG RNA labeling mix, Roche Diagnostics).

*2.3. Sequencing of PSTVd progeny ~~in~~ from infected plants*

Total RNAs from 100 mg of infected tomato leaves or roots were extracted using the RNeasy Plant Mini Kit (Qiagen) following manufacturer’s instruction and used in RT-PCR reactions to amplify full-length PSTVd cDNAs. The reactions were performed using two specific primers corresponding to the CCR region (Góra et al., 1994) and the Omniscript Reverse Transcriptase kit (Qiagen) and Easy-A High-Fidelity PCR Master Mix (Stratagene). The PCR products were directly sequenced and the obtained sequences analyzed using Lasergene software (DNASTAR Inc.). When sequence heterogeneity was observed, the PCR products were cloned into the pGEM T-Easy vector (Promega) and 4-6 clones re-sequenced.

*2.4. Prediction of PSTVd RNA secondary structure*

The predicted secondary structures of the circular PSTVd RNA at 370C in 1M NaCl were computed using the Mfold 3.2 program (Zuker, 2003).

*2.5. Haplotype network analysis*

The sequences of 27 variants of Ava-del mutants and their progeny were aligned using ClustalW (<http://www.genome.jp/tools/clustalw/>). A parsimony haplotype network analysis was conducted using the TCS 1.21 program with connection limit set to 95% (Clement et al., 2000).

**3. Results**

*3.1. Characterization and infectivity of the ~~constructed~~ PSTVd indel mutants*

To observe the influence of sequence modification on viroid infectivity and pathogenecity the PSTVd-S23 variant was chosen as the parental molecule for mutagenesis. Its high infectivity and genetic stability and the induction of characteristic severe disease symptoms make this variant a very convenient tool (Góra-Sochacka et al., 1997; Więsyk et al., 2011). Indel mutants were obtained using three unique restriction sites of the viroid cDNA molecule (Table 1). The *Eag*I site is located at the border of the V and TR domains on the upper strand between nucleotide positions 144-149 (numbering of the parent PSTVd-S23, Fig. 1a). The *Ava*II site (positions 220-224) and the *Sty*I site (positions 336-341) are located on the lower strand of the V and TL domains, respectively (Fig. 1a). All introduced mutations are predicted to modify the local secondary structure (Fig. 1b) and were verified by Sanger sequencing.

*Argobacterium tumefaciens* cells transformed with the recombinant pGreen vectors containing the various mutant cDNAs were used to agroinoculate tomato seedlings (25 plants for each construct). The accumulation of PSTVd progeny was evaluated by dot-blot hybridization four and eight weeks after agroinoculation. None of the plants inoculated with the indel mutants affecting the *Eag*I and *Sty*I sites generated hybridization signals nor expressed disease symptoms, showing that these mutants are non-infectious. By contrast, 44% (11) and 84% (21) of those plants inoculated with the Ava-del mutant showed detectable PSTVd progeny at 4 and 8 weeks post inoculation (wpi), respectively. Similar results were obtained for the Ava-in mutant, with 40% (10/25) and 80% (20/25) infected plants at 4 and 8 wpi, respectively. The appearance and severity of symptoms were compared to those of plants inoculated with the parental PSTVd-S23, all of which expressed the first disease symptoms 2 wpi. In contrast, the first characteristic symptoms were observed no sooner than 4 wpi on PSTVd-Ava-in and -del infected plants. Plants inoculated with the Ava-in mutant displayed, epinasty, growth inhibition and necrosis on stems and leaves, albeit plants were higher than those infected with PSTVd-S23. The symptoms observed on plants inoculated with the Ava-del mutants were severe (such those observed for PSTVd-Ava-in) or intermediate, depending on the viroid population in the infected plants (Table 2).

*3.2. Analysis of the progeny of PSTVd-Ava indel mutants*

*3.2.1. PSTVd-Ava-in*

The progeny of the PSTVd-Ava-in was analyzed at the two time points after agroinfiltration, in 9 of the infected plants. At the early stage of infection (4 wpi) in 5 of the plants the PSTVd-Ava-in mutant was the only sequence detected while in the other 4 plants a mixture of the mutant with the parental wild-type PSTVd-S23 was observed. Three weeks later, PSTVd-S23 was the dominant sequence (data not shown). These results suggest that, even if viable, the PSTVd-Ava-in mutant is unstable and reverts rapidly toward the parental wild-type PSTVd-S23 sequence.

*3.2.2. PSTVd-Ava-del*

Contrary to PSTVd-Ava-in, the progeny of PSTVd-Ava-del, also analyzed in 9 plants, formed more complex populations (Table 2). In one plant (No 2) reversion of the GAC deletion (positions 221-223) was observed at 5 wpi. However, analysis of the viroid progeny in the other 8 analyzed plants revealed new sequence variants with alterations at and outside the *Ava*II deletion site and reversion to the parental S23 variant was not observed. In total, ten different variants deriving from PSTVd-Ava-del mutant (Ava-del-1, -2, -3, -4, -5, -6, -7, -8, -9, -10) were observed in the progeny of infected plants (Table 2). Seven of these variants (-1, -2, -3, -4, -5 -6 and -10) conserved the initial 221GAC223 deletion, while three others (-7, -8 and -9) incorporated three nucleotides at these positions, but ~~a~~ CAG instead of the original GAC sequence (Table 3).

Variants Ava-del -3, -6, -9, -10 were only observed at early time points. Variant Ava-del-2 was observed in one plant only at the late time point and in 4 plants at early time points and was not observed again in the three plants (9, 14 and 16) that were reanalyzed later (Table 2).

*3.3. Molecular analysis of the progeny variants of PSTVd-Ava-del*

The ten sequence variants (Ava-del -1, -2, -3, -4, -5, -6, -7, -8, -9, -10) recovered in the progeny of the PSTVd-Ava-del mutant (Table 2) were subjected to further analysis focusing on their predicted secondary structures, virulence and genetic stability.

*3.3.1. Secondary structure predictions*

As shown in Fig. 1c the predicted local secondary structure of the variants that retained the GAC three nucleotides deletion at the *Ava*II site is very similar or identical to that of the PSTVd-Ava-del mutant. The exception are two variants, Ava-del-1 with an additional dinucleotide deletion (141UA142) and Ava-del-2 with the G137A substitution. The secondary structure of the sequence variants that incorporated the three nucleotides 221CAG223 (instead of the original GAC) is different from the parental PSTVd-Ava-del structure and also from that of the parental wild type PSTVd-S23.

*3.3.2. Pathogenicity and genetic stability*

Tomato seedlings were inoculated (via agroinfiltration) with the various PSTVd-Ava-del progeny sequence variants and at an early stage of infection (3 or 5 wpi, depending on the symptom development) their progeny were examined in the upper leaves of symptomatic plants. A second round of progeny analysis was also performed a few weeks later, and involved both leaf and root tissues (Table 3).

At 3 wpi dot blot hybridization confirmed the infectivity of all analyzed variants, except Ava-del-2, -9 and -10 (data not shown). At this time point only the sequence of the inoculated variant was observed in the infected plants. Two weeks later (5 wpi) samples from plants inoculated with Ava-del -2 and -9 gave positive signals in dot blot hybridization and sequence analysis showed that the viroid populations harbored mutations outside of the *Ava*II site (Table 3). Based on the lack of hybridization signals up to 8 wpi (data not shown), Ava-del-10 was considered non-infectious.

With one exception (Ava-del-1) analysis of progeny of other viable variants derived from the PSTVd-Ava-del mutant indicated sequence diversity at the late sampling time point (8 wpi, Table 3). To clarify the mutational events and phylogenetic correlations between all detected sequence variants, a network of 26 detected haplotypes (including 10 parental PSTVd-Ava-del variants and their progenies) was reconstructed using on parsimony analysis (Fig. 2) (Clement et al., 2000). Ava-del-2 variant revealed a very low infectivity (2/10 inoculated plants, Fig.1c), which may explain why it was mostly observed as a transient variant detected at early time points following agroinoculation of the Ava-del mutant (Table 2). The accumulation of further mutations follows several pathways, some of which are repeatedly observed in the haplotype network, in particular the gain of the 221CAG223 insertion leading to Ava-del-7 and also seen in Ava-del-2 progeny or the UA141-142 deletion leading to stable Ava-del-1 or seen in the Ava-del-2 progeny (Fig. 2). Moreover, the G137A substitution leading to Ava-del-2 is the most frequently observed and stably maintained mutation in the populations deriving from Ava-del. This substitution is located on the upper strand above the *Ava*II site and influence loop 20 structure, the closing of which is known to lead to defective trafficking and to a decrease of replication efficiency (Zhong et al., 2008).

Ava-del-7 is only one mutational step away from Ava-del-9, Ava-del-8 and its progeny *pAva-8.3*. Further simple pathways of mutations accumulations can account for the diverse progenies observed for these three mutants (Fig. 2), which all share the same insertion of three nucleotides at *Ava*II site (221CAG223, in opposite orientation than in the parental PSTVd-S23), with ~~sometimes~~ occasional secondary mutations affecting the inserted nucleotides; i.e. GAG, GAA or GAU (*pAva-7.2, -8.2, -8.4, -9.2* and *-9.3*).

Variant Ava-del-6 differs from Ava-del-5 only by the substitution U246C, and its progeny, which contains an additional C insertion between nucleotides 229 and 230, is closely connected to both Ava-del variants, forming another mutational network initiated by mutation A225G and centered on Ava-del-5. Lastly, variant Ava-del-3 differing from Ava-del mutant by ~~a~~ the C129U substitution was highly infectious; however, an additional substitution (C337A) resulted in transition to non-infectious variant Ava-del-10. Ava-del-4 differs from Ava-del-3 by two nucleotide substitution A30G and C138U but their progenies tend to converge towards the same C138U+ C129U variant (Fig.2).

**4. Discussion**

Of the six PSTVd indel mutants analyzed here, four (Eag-del, Eag-in, Sty-del and Sty-in) were nonviable. This result fits with the observed pattern of sequence variability in the PSTVd genome. Alignment of the 333 PSTVd sequences deposited in GenBank showed the lowest variability at the mutated *Sty*I site (nt 336-341), with only two recorded variants with mutations at these positions (substitutions 339UU340/AU or /GC) (Lakshman and Tavantzis, 1992).

In the present study, insertion or deletion of four nucleotides resulted in non-infectious mutants. The *Sty*I site is located in loop 4 within the TL domain, which play a crucial role in viroid replication (Bojic et al., 2012; Kolonko et al., 2006). The insertion is predicted to lead to the replacement of an existing loop by two small loops (Fig. 1b). The deletion results in enlargement of the upper strand bulge by C21 and loosing the bulge on the lower strand. Closing loop 4 has been shown to reduce replication to below 16% of the wild-type level (Zhong et al., 2008). Moreover, transcription factor TFIIIA-7ZF, which aids PSTVd transcription *in planta*, binds to PSTVd RNA in a region spanning nucleotides 331-347 and covering loop 3, 4 and 5 (Wang et al., 2016). ~~In this respect, it is noteworthy that~~ Variant PSTVd-Ava-del-10 also confirms the important role of this region: substitution C337A is predicted to minimize loop 4 to two U following the paring of G23:U338 and U24:A337 (Fig. 1c), possibly rendering Ava-del-10 non-infectious.

Mutations at the *Eag*I site (144-149) are observed more frequently in PSTVd natural variants, with seven different variant haplotypes (substitutions or deletions) observed in 13 sequence variants. In comparison, five haplotypes are observed at the *Ava*II site (220-224) in a total of 42 variants. Mutant Eag-del has been described previously (Lakshman and Tavantzis, 1992) and similarly found non-infectious. Closing of loops 21-23 in this region affects negatively replication and trafficking (Zhong et al., 2008). Indel mutations in the *Eag*I site lead to a major rearrangement of the secondary structure (Fig. 1a and b). The *Eag*I site is located next to the internal RY motif, which together with the external RY motif, is involved in binding of Virp1 protein (Gozmanova et al., 2003) involved in systemic spread of PSTVd or in RNA-mediated DNA methylation (Kalantidis et al., 2007; Maniataki et al., 2003). Structural changes may negatively affect the RY-mediated binding of Virp1 resulting in inhibition of viroid infection cycle.

In comparison with the *Sty*I and *Eag*I mutants, alterations at the *Ava*II site involve only three nucleotides indels, which may facilitate the repair process. The PSTVd-Ava-indel mutants both proved viable but genetically unstable. Repair of the three nucleotides indel mutations is noteworthy and raises questions about viroid ‘self-repair’ capacity. It is known that maintenance of the native secondary structure plays a key role in viroid viability (Hammond, 1994; Hammond and Owens, 1987; Hu et al., 1997; Qu et al., 1993; Wassenegger et al., 1994).

Previous results (Qin et al., 2014) indicate that 20.7% (74 nucleotides) of PSTVd-S23 genome are comprised of simple sequence repeats (SSR), with trinucleotide repeats occuring with the highest frequency. SSRs could play an important role in viroid evolution and could lead to sequence diversification (or stability) where insertion-deletion mutations play an important role. Strand-slippage mechanism may play a main role in deletion-insertion mutagenesis; this mechanism could be expected to occur frequently in viroid replication because the processivity and fidelity of the DNA-dependent RNA polymerase is probably affected when it is forced to transcribe viroid RNA templates (Acosta-Leal et al., 2011; Qin et al., 2014). Moreover, polymerase slippage is positively correlated with the length of repeated sequence and even very short repeats (two copies) exhibit a higher frequency of insertions and deletions than non-repetitive sequences (Gressel and Levy, 2006; Taylor et al., 2004). Also insertions and deletions are more common in higher G+C content sequences (Taylor et al., 2004). In this context it is noteworthy that in the Ava-del mutant there is a 217GCA/GCA222 repetition. Polymerase slippage would introduce an extra three GCA nucleotides, thus mimicking the insertion observed in variants Ava-del -7, -8 and -9 as well as in some of the progenies of variants Ava-del -2 and -5 (Fig. 2 and 3). The reversion of the Ava-in mutation to the wild type S23 sequence involves the loss of three nucleotides. These nucleotides are duplicated in the mutant sequence (221GAC/GAC226) which may facilitate the simple deletion of one repetition (Gressel and Levy, 2006). Moreover, based on sequence similarity on the upper strand or on the presence of complementary sequences like the inverted nucleotide stretch 197GGGUGUCCU205 and 219AGGACCACCC228, a polymerase switch may enable removal of these three extra nucleotides (Gressel and Levy, 2006).

Leaves and roots differ in anatomical structures, cell composition, pattern of gene expression and are exposed to different environmental condition (Andika et al., 2016). These differences could potentially influence viroid populations but on the other hand PSTVd moves systemically from source to sink and from roots to shoot apex (Mehle et al., 2014; Wang and Ding, 2010), which may result in mixing both population. In the experiment reported here, the composition of the viroid population in roots and leaves did not differ drastically (Table 3). However, the population observed in leaves was more diverse, because sequencing of 43 cDNA clones revealed 20 haplotypes (0.46 haplotype/clone) while 15 haplotypes/41 clones (0.36/clone) were observed in roots. As shown on Fig. 2 six haplotypes were leaf-specific but only one was specific to roots.

In conclusion, two of six created indel mutants in loops of the PSTVd rod-like secondary structure were viable but genetically unstable. Observed repair of trinucleotide deletion or insertion was facilitated by occurrence of trinucleotide repeats in the region of mutations. Analysis of progeny of viable variants derived from the constructed PSTVd-Ava-del mutant indicated more diverse population in leaves than roots. It should be stressed, however, that our analysis of PSTVd mutants progenies was directed towards the detection of sequences representing a significant proportion of the population (not deep population analysis), so that further studies will be needed to reach more firm conclusions.Finally, we reconstructed network of all detected haplotypes which allows to understand the observed mutation events and to clarify phylogenetic correlation between all detected sequence variants.

**Acknowledgment**

We thank Wojciech Podstolski for generation of some mutations in the PSTVd cDNA cloned in pUC9 vector, and Sylwia Kacprzak for technical assistance.

**Funding**

This research did not received any specific grant from funding agencies in the public, commercial, or not-for-profit sectors

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**Figures and Tables**

**Fig. 1.** Predicted secondary structure of the PSTVd RNA. (a) Secondary structure of the full-length PSTVd-S23 genome, 358nt. TL, P, C, V and TR – structural domains: Terminal left, Pathogenic, Central, Variable and Terminal right, respectively. Nucleotides in orange, red and blue display the *Sty*I, *Ava*II and *Eag*I restriction sites, respectively. Loops are numbered according to Zhong *et al.* (Zhong et al., 2008). Loop 16 in the PSTVd-S23 variant is split into 16a and 16b. (b) Local (around mutated sites) secondary structure of the six engineered PSTVd mutants. Inserted nucleotides are underlined. (c) Local secondary structure of the 10 progeny variants observed in plant after PSTVd-Ava-del inoculation. Additional mutations (outside *Ava*II site) are indicated in green. \*Symptoms observed at 5 wpi. #Number of infected over number of inoculated plants.



**Fig. 2.** Network of haplotypes observed during the study. The network was reconstructed using the TCS program and all identified haplotypes, excluding PSTVd-S23. It was then simplified according to experimental data. In italics variants observed in the progeny of Ava-del variants. Sequences observed only in leaves are shaded, root-specific sequence is marked by asterisk.

**Fig. 3.** Proposed mechanism of CAG insertion. Nucleotides of the *Ava*II restriction site are bolded. Three nucleotides GCA repetition are boxed.

212C G C C C G C A **G G A C C** A C C C C U C G C233PSTVd-S23, wild type

212C G C C C G C A **G \_ \_ \_ C** A C C C C U C G C233PSTVd-Ava-del mutant

212C G C C C G C A **G C A G C** A C C C C U C G C233PSTVd-Ava-del-7, -8, -9 variant

**Table 1.** Sequence of the targeted restriction sites before and after mutagenesis.

Nucleotides inserted or deleted during mutant construction are underlined or shaded, respectively. Nucleotide numbering according to the PSTVd-S23 sequence. \*total length (in nucleotides) of PSTVd mutant genome.

|  |  |  |  |
| --- | --- | --- | --- |
| **Mutant name** | **Targeted sequence** | **Mutated sequence** | **Length (nt)\*** |
| PSTVd-Eag-in | 144C G G C C G149 | ..C G G C C G G C C G.. | 362 |
| PSTVd-Eag-del | 144C G G C C G149 | ..C G.. | 354 |
| PSTVd-Ava-in | 220G G A C C224 | ..G G A C G A C C.. | 361 |
| PSTVd-Ava-del | 220G G A C C224 | .. G C.. | 355 |
| PSTVd-Sty-in | 336C C U U G G341 | ..C C U U G C U U G G.. | 362 |
| PSTVd-Sty-del | 336C C U U G G341 | ..C G.. | 354 |

**Table 2**. Progeny of the constructed PSTVd-Ava-del mutant in particular plants.

Sequence frequency is expressed as the number of cDNA clones with the given sequence variant per number of sequenced clones and is provided in brackets. PCR, sequence homogeneity was observed on RT-PCR product.

|  |  |  |
| --- | --- | --- |
| **Plant No** | **Wpi** | **Name of sequence variants** |
| 2 | 5 | S23 (PCR) |
| 1, 18 | 6 | Ava-del-5 (PCR) |
| 5 | 4 | Ava-del (2/4), Ava-del-6 (2/4) |
| 5 | 7 | Ava-del-5 (PCR) |
| 9 | 4 | Ava-del (2/4), Ava-del-2 (2/4) |
| 9 | 7 | Ava-del-7 |
| 14 | 5 | Ava-del-7 (2/5), Ava-del-2 (1/5), Ava-del-10 (1/5), Ava-del-3 (1/5) |
| 14 | 8 | Ava-del-7 (2/4), Ava-del-8 (1/4), Ava-del-4 (1/4) |
| 16 | 5 | Ava-del-7 (2/4), Ava-del-2 (1/4), Ava-del-9 (1/4) |
| 16 | 8 | Ava-del-7 (4/5), Ava-del-1 (1/5) |
| 19 | 6 | Ava-del (PCR) |
| 19 | 8 | Ava-del (2/4), Ava-del-2 (2/4) |
| 20 | 4 | Ava-del (2/4), Ava-del-2 (2/4) |

**Table 3.** PSTVd-Ava-del sequence variants and their progeny.

Sequence frequency (after 8 wpi) is expressed as the number of cDNA clones with the given sequences per number of sequenced clones and is provided in brackets. At early stage of infection (3 wpi and 5wpi) PCR products were directly sequenced (heterogeneity was not observed).

|  |  |  |
| --- | --- | --- |
| **Parental variant** | **Mutation** | **Nucleotide difference of progeny from the parental sequence** |
| **Leaves** | **Roots** |
| ***Ava*II site** | **Outside** | **3 wpi** | **8 wpi** | **8 wpi** |
| **Ava-del-1** | ∆221GAC223 | ∆141UA142, G137A | Ava-del-1 | Ava-del-1 (4/4) | Ava-del-1 (4/4) |
| **Ava-del-2** | ∆221GAC223 | G137A | *pAva-2.1*: C164A, C166U **5wpi** | *pAva-2.1* (1/5)*pAva-2.2*: ∆141UA142, C164A, C166U (1/5)*pAva-2.4*: +221CAG223, C164A, C166U, G201U (3/5); | *pAva-2.1* (3/5)*pAva-2.3*:+221CAG223, C164A, C166U (2/5); |
| **Ava-del-3** | ∆221GAC223 | C129U | Ava-del-3 | *pAva-3.1*:C138U (3/4)S23 (1/4) | *pAva-3.1* (3/4)S23 (1/4) |
| **Ava-del-4** | ∆221GAC223 | A30G, C129U, C138U | Ava-del-4 | *pAva-4.1*: G30A (4/4) | *pAva-4.1*: G30A (4/4) |
| **Ava-del-5** | ∆221GAC223 | A225G | Ava-del-5 | Ava-del-5 (2/5)*pAva-5.1*: +221CAG223 (2/5); S23 (1/5) | Ava-del-5 (2/5)*pAva-5.1* (3/5) |
| **Ava-del-6** | ∆221GAC223 | A225G, U246C | Ava-del-6 | Ava-del-6 (2/5); Ava-del-5 (1/5);*pAva-6.1*: +C (229-230) (1/5)*pAva-6.2*: C246U, +C (229-230) (1/5) | Ava-del-6 (2/5); Ava-del-5 (1/5); *pAva-6.1* (1/5)*pAva-6.2* (1/5) |
| **Ava-del-7** | +221CAG223 | G137A | Ava-del-7 | Ava-del-7 (3/5)*pAva-7.1*: C221G,U136A (1/5)*pAva-7.2*: C221G, G223A (1/5) | Ava-del-7 (5/5) |
| **Ava-del-8** | +221CAG223 | G137A, C216A | Ava-del-8 | Ava-del-7 (2/5)*pAva-8.1*: C221G, G223A, G201U (1/5);*pAva-8.2*: C221G, G223U, G201U (1/5)*pAva-8.3*: C221G; A216C (1/5) | Ava-del-7 (3/5)*pAva-8.3* (1/5);*pAva-8.4*: C221G, G223A, A216C(1/5) |
| **Ava-del-9** | +221CAG223 | G137A, G211A | *pAva-9.1*:C148U **5wpi** | *pAva-9.1* (2/6)*pAva-9.2*: C221G, C148U (2/6);*pAva-9.3*: C221G, G223A, C148U (2/6) | *pAva-9.1* (2/4);*pAva-9.2* (2/4) |