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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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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.

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

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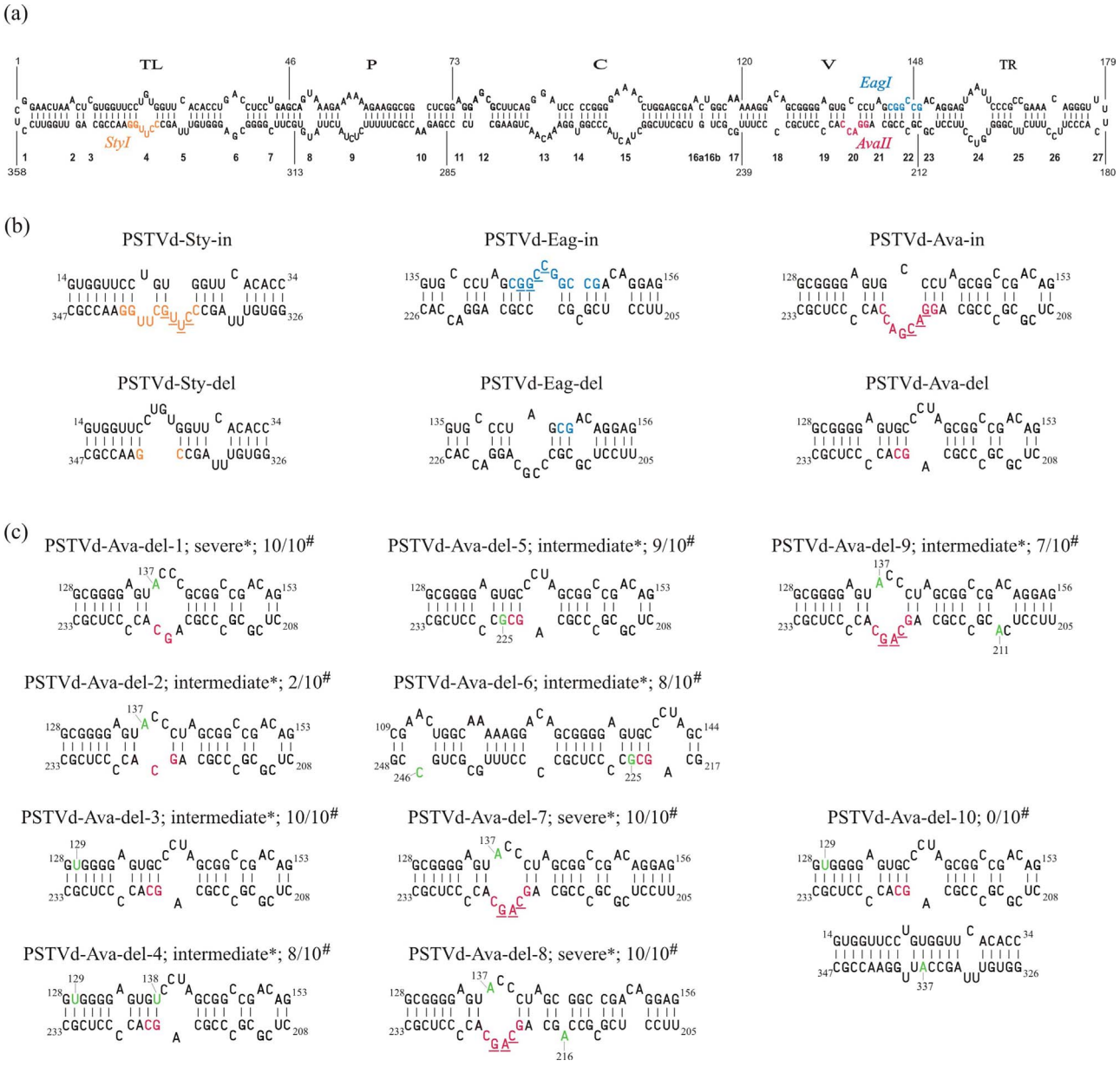

Fig. 1. Predicted secondary structure of the PSTVd RNA. (a) Secondary structure of the full-length PSTVd-S23 genome, 358nt. TL, P, C, V and TR − structural domains: Terminal left, Pathogenic, Central, Variable and Terminal right, respectively. Nucleotides in orange, red and blue display the *Sty*I, *Ava*II and *Eag*I restriction sites, respectively. Loops are numbered according to Zhong et al. (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. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

et al., 2004); ii) loop 7 (U43/C318), which enables movement from non-vascular to phloem tissue (Zhong et al., 2007); iii) loop 6 (36CG-A38/323GAC325 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