

SHORT COMMUNICATION

ANALYSIS OF SYMPTOMS DEVELOPED IN *NICOTIANA BENTHAMIANA* PLANTS EXPRESSING DIMERIC FORMS OF *HOP STUNT VIROID*

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SUMMARY

Viroids are sub-viral plant pathogens consisting of covalently closed circular RNAs that infect herbaceous and woody hosts. They do not code for any peptide or protein and therefore are fully dependent on the host machinery for most biological processes, including pathogenesis. Certain viroid-host combinations show severe symptoms but the biochemical bases of this process are currently unknown. In this work, we have characterized the symptoms induced by *Hop stunt viroid* (HSVd) when provided as a transgene in *Nicotiana benthamiana* plants, a natural non-host that is able to process and translocate HSVd. The presence of HSVd in this transgenic plant causes severe stunting and decrease in the leaf size, followed by strong alteration of flower development and reduced fertility. These symptoms are similar to those induced by HSVd in natural infections, indicating that in *N. benthamiana* HSVd expression mimics the metabolic disorders induced in natural hosts. Therefore, these transgenic plants may be useful to study the viroid-host interactions leading to pathogenesis.

Key words: viroid-host interaction, hop stunt, transgenic plants, pathogenesis.

Intimate host-pathogen interactions can result in pathogenesis, depending on the host species, pathogen variants and environmental conditions. With viroids, interdependence with host factors reaches its maximum level. Viroids are plant pathogens whose genome is composed of a small (250-400 nt) single-stranded circular RNA molecule that does not have protein coding capacity but is able to replicate in susceptible host plants (Tabler and Tsagris, 2004; Flores *et al.*, 2005a). Viroid species are classified into two families, *Pospiviroidae* and *Avsunviroidae* (Flores *et al.*, 2005b). Viroids in the family *Pospiviroidae* contain a central conserved region

(CCR) in their sequence and replication takes place in the nucleus. On the other hand, members of the family *Avsunviroidae* lack a CCR, replicate in the chloroplast and are able to self-cleave through hammerhead ribozymes (Flores *et al.*, 2005a). Given that viroids have no ability to encode any pathogen-specific protein, their propagation and associated pathogenesis are fully dependent on the host biochemical machinery. The interaction activates a sequence of poorly understood events that are expressed as visible symptoms. How these pathogenic RNAs interact with cellular factors to modify host development and physiology is an essential question about the viroid-induced pathogenesis. The most typical symptoms are stunting and the alterations in flowering (Sano, 2003), usually characterized by shortening of internodes, reduced flower and leaf size and fruit alterations (Diener *et al.*, 1988; 1989; Shikata *et al.*, 1990; Amari *et al.*, 2001).

Hop stunt viroid (HSVd) is the type species of the genus *Hostuviroid* in the *Pospiviroidae* family. Variants of HSVd have been identified in several crops in which they may or may not cause disease (Shikata, 1990; Kofalvi *et al.*, 1997; Amari *et al.*, 2007). Attempts to transmit by conventional inoculation HSVd to *N. benthamiana*, a model plant to study plant-pathogen interactions, failed. Recently we have reported that *N. benthamiana* possesses the machinery for processing HSVd and the cellular factors required for its long distance translocation, emerging as an important instrument to perform plant-viroid interaction studies (Gómez and Pallás, 2006). In addition, it was described that the presence of biological forms of HSVd could be associated with development of alterations.

In the present work we have characterized the pathogenic effects associated with HSVd in *N. benthamiana*, specifically the alterations in growth and flowering since these characters are the most frequent disorders produced by HSVd in naturally infected plants. In this study, we used transgenic *N. benthamiana* plants expressing a dimeric form of HSVd (*HSVd-Nb*) that have the capability to cleave and circularize the viroid RNA (Gomez and Pallas, 2006). Seeds of two lines of *HSVd-Nb* plants (lines 6 and 8), were germinated and maintained for 4 weeks in climate chambers at 20°C and later on moved to a glasshouse at 28°C with supplementary

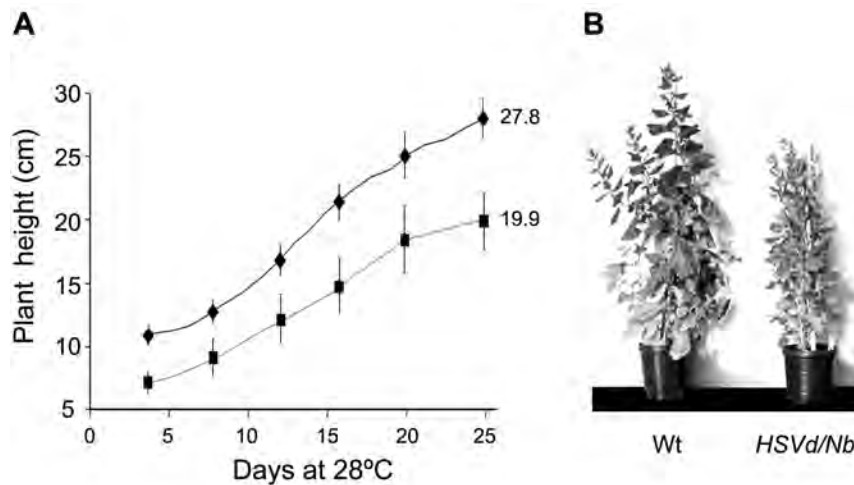


Fig. 1. Influence of HSVd on the dynamics of *N. benthamiana* growth. **A)** Effects of the presence of HSVd biological forms on the development of transgenic *N. benthamiana* plants (◆). Plant height was measured at 4, 8, 12, 16, 20 and 25 days after incubation at 28°C. Untransformed *N. benthamiana* (■) used as control. At 25 days, the size of the *HSVd-Nb* plants corresponded in average to 72% (19.9 cm) of the untransformed plants (27.8 cm). **B)** General symptoms induced by HSVd in a representative transgenic *N. benthamiana* plant compared with an untransformed control at 25 days.

illumination to maintain a 16-h daylight period (hereafter all data will be referred to the incubation at 28°C).

These are the optimal conditions for expression of HSVd symptoms in natural hosts. Twelve *HSVd-Nb* plants accumulating similar levels of monomeric circular and linear HSVd-forms were used in each experiment with three replicates. The times for measurement of the different physiological parameters are given in the respective figure legends. *HSVd-Nb* plants showed symptoms 4 days after incubation at 28°C. Significant stunting was observed in *HSVd-Nb* plants compared with untransformed *N. benthamiana* (Fig. 1A). Stunting was maintained throughout the incubation period (Fig. 1A) and was correlated with shortening of the stem internodes. Although slight differences were observed between the two transgenic lines, data were obtained from analysis of 24 characterized plants of the two lines (12 plants each). At 25 days, transformed plants rendered, on average, 72% of the plant height of the corresponding untransformed plants (Fig. 1A and B). *Nicotiana benthamiana* plants transformed with an empty vector and used as controls to eliminate transgene effects showed normal development (data not shown).

Another characteristic symptom of HSVd infection is alteration of flowering leading to deficiencies in fruit quality and seed viability (Singh *et al.*, 2003). To determine if HSVd induces similar symptoms in *N. benthamiana*, we analyzed different aspects of flowering such as appearance of the first flower or number of flowers and fruits per plant (Fig. 2). The flowering of transgenic plants was delayed 7 days in comparison to the control plants, independently of the transgenic line analyzed (Fig. 2A). Analysis of the number of flowers and fruits revealed a negative effect of the presence of

HSVd-RNA. The total number of flowers at 10, 17 and 24 days after the initiation of flowering corresponded respectively to 12%, 21% and 45% of the control plants (Fig. 2A), indicating both a delay in flowering and a significant reduction in flower number.

A reduction of flower size is another morphological character observed in HSVd natural infections in hops (Sano, 2003) or cucumbers (Van Dorst and Peters, 1974). Flower length in each *HSVd-Nb* transgenic line was measured at the beginning of ripening after 60 days at 28°C. Sixty flowers of each transgenic line and untrans-

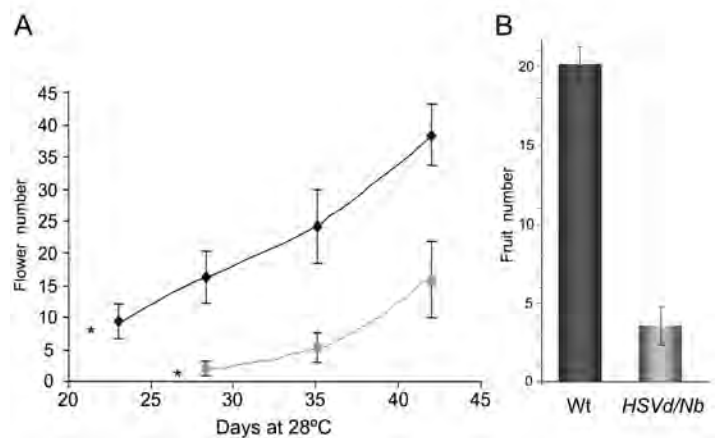


Fig. 2. Analysis of flowering in *HSVd/Nb* plants. **A)** Flower number in the *HSVd/Nb* plants (◆) and untransformed controls (■), counted at 3, 10, 17 and 24 days after flowering began (*) in control plants. In *HSVd/Nb* plants, flowering was delayed 7 days compared with untransformed *N. benthamiana*. **B)** Fruit number was determined after 60 days at 28°C. The number of fruits in *HSVd/Nb* plants was on average 16% less than in untransformed controls.

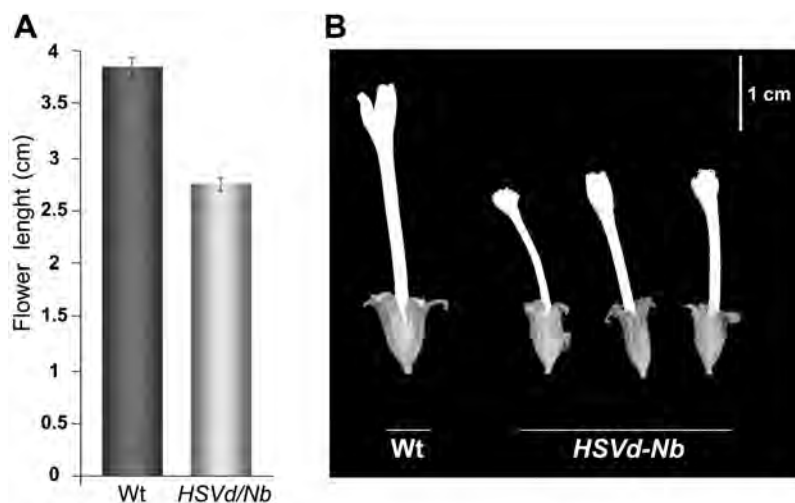


Fig. 3. Effects of HSVd on flower development in *N. benthamiana* plants. **A)** Flower length of *HSVd/Nb* plants was measured at the beginning of ripening after 60 days at 28°C, and compared with flowers of untransformed plants. The *HSVd/Nb* flowers were on average approximately 80% of the length of control plant flowers. **B)** General aspect of representative flowers of *HSVd/Nb* plants compared with a control flower.

formed *N. benthamiana* controls were analyzed. The average size of flowers of *HSVd-Nb* plants was 81% of the size of the wild type (Figs 3A and 3B), in accordance with that observed in HSVd natural infections. Furthermore, the affected flowers aborted before ripening, resulting in a severe decrease of fruit number (Fig. 2B). As a consequence of flowering and fruit alterations, seed production was reduced in number and quality (data not shown). This decrease of fertility in *HSVd-Nb* plants was previously reported (Gómez and Pallás, 2006). In addition, it was recently suggested that the sterility observed in infected wild chamomile species is probably associated with viroid pathogenesis (Matoušek *et al.*, 2007a).

The results obtained indicate that the presence of HSVd forms (circular and linear) in transgenic *N. benthamiana* is associated with a “pathogenic” process characterized by stunting. A slight decrease in leaf size in transformed plants was also observed (data not shown). This growth disorder was accompanied by strong alteration of flower development and reduced fertility. The symptoms observed in *HSVd-Nb* plants are coincident with the most common disorders induced by HSVd in their natural hosts (Diener *et al.*, 1988; Shikata *et al.*, 1990). Apparently, the pathogenic processes associated with the presence of biological forms of HSVd in transgenic *N. benthamiana* mimic the disorders in unidentified metabolic pathways induced by conventional viroid infection. This observation is in accordance with a recent report indicating that biolistic transfer of a *Potato spindle tuber viroid* (PSTVd) strain to *N. benthamiana*, (a symptomless PSTVd host), led to a strong pathogenic reaction, suggesting the presence of specific viroid pathogenesis-promoting target(s) in this plant (Matoušek *et al.*, 2007b).

Although the mechanism of viroid-induced patho-

genesis is not clear, different hypotheses have been proposed. The most attractive idea involves specific host-mRNA degradation mediated by viroid-induced RNA silencing (Wang *et al.*, 2004). This hypothesis has been reinforced by recent work demonstrating the ability of two members of the *Pospiviroidae* family, PSTVd and HSVd, to simultaneously elicit and resist this plant defense mechanism in tomato (Itaya *et al.*, 2007) and *N. benthamiana* (Gómez and Pallás, 2007) plants. In addition, the *HSVd-Nb* lines analyzed here accumulated HSVd-specific siRNAs (Gómez and Pallás, 2007). A more detailed study on the possible correlation between HSVd-specific accumulation of siRNAs and symptom expression in transgenic *N. benthamiana* plants is currently in progress. The alternative possibility that the mature viroid molecule, derived from processing and circularization of the transgenic transcript, could also be responsible for the symptoms observed in the transgenic lines, cannot be ruled out. In any case, the model *HSVd-N. benthamiana* will be an attractive system where both RNA silencing and HSVd-induced pathogenesis take place, providing a valuable tool to study the specific host-viroid interactions involved in pathogenesis.

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