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The molecular characterization of HSVd isolates associated with dapple fruit and fruit rugosity in plum seedlings suggests a possible role of breeding in viroid dissemination

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Abstract

In a wide range of hosts, the infection caused by *Hop stunt viroid* (HSVd) appears to be latent, whereas in some others it is frequently pathogenic. In this work, the presence of HSVd has been found to be associated with symptoms of dapple fruit and fruit rugosity in plum seedlings obtained from cross breeding for quality. Symptomatic and symptomless plum seedling samples have been analyzed for the presence of the principal stone fruit viroids and viruses. HSVd was found in all symptomatic samples, whereas no other viruses or viroids were found in the analyzed samples with the exception of ACLSV, which was detected rarely. The RNAs of all HSVd isolates have been cloned and sequenced. The sequence analysis showed a high percentage of homology among the isolates, making it possible to hypothesize a potential unique origin of the infection. For this purpose, those plants used in breeding as pollen donors have been analyzed. The results showed that the same HSVd isolate was also present in the parental plants, both in the leaves and pollen, suggesting a possible role of breeding in the dissemination of the viroid.

Keywords: plum, seedlings, fruit rugosity, dapple fruit, HSVd, pollen

Introduction

Viroids are subviral pathogens consisting of a circular single strand of RNA that ranges in size from 240 to 400 nucleotides. Their genome is highly structured without any detectable messenger activity so they are host-dependant in their life-cycle (Diener, 1991). Viroids are classified into two families: *Pospiviroidae* and *Avsunviroidae* (Flores et al., 1998). *Hop stunt viroid* (HSVd) belongs to the *Hostuviroid* genus, itself belonging to the *Pospiviridae* family, with a size that ranges between 294-303 nt. HSVd has been found in a wide variety of hosts: hop, cucumber, grapevine, citrus, plum, peach and pear (Shikata, 1990) as well as apricot and almond (Astruct et al., 1996, Canizares et al, 1999). In some species, like grapevine, the infection seems to be latent (Shikata, 1990; Polivka et al., 1996). Contrastingly, in some others, like hop, citrus, plum, peach and apricot, certain variant sequences can cause symptoms like stunting, dapple fruit, fruit rugosity and cachexia (Diener et al., 1988; Sano et al., 1989, Shikata, 1990, Ragozzino et al., 2002 , Amari et al., 2007).

The variants in plum and peach induce the dapple fruit disease that was first discovered in Japan in 1986 on *Prunus salicina* 'Taiyo' (Terai, 1985). Symptoms of dapple fruit disease are restricted to the fruit and they vary according to species (peach or plum) and to cultivars. Generally, it induces chlorotic blotches and spots on the skin of the fruit, and the surface of the fruits also becomes irregular (Sano et al., 1989). In Italy, HSVd was reported on symptomless stone fruit samples by Loreti *et al.* (1998). Subsequently, dapple fruit disease was reported by Ragozzino et al. (2002) in Japanese plum 'Sorriso di Primavera' and 'Florentia'.

In this work, plum seedlings, obtained from cross breeding for quality and showing dapple fruit and fruit rugosity symptoms (Figure 1), have been analyzed for the presence of viroids (HSVd and *Peach latent mosaic viroid* – PLMVd) and viruses (*Plum pox virus* – PPV, *Apple chlorotic leaf spot virus* – ACLSV, *Prunus dwarf virus* – PDV, *Prunus necrotic virus* – PNRSV, *Strawberry latent ring spot virus* – SLRSV, *Cherry leaf roll virus* – CLRV).

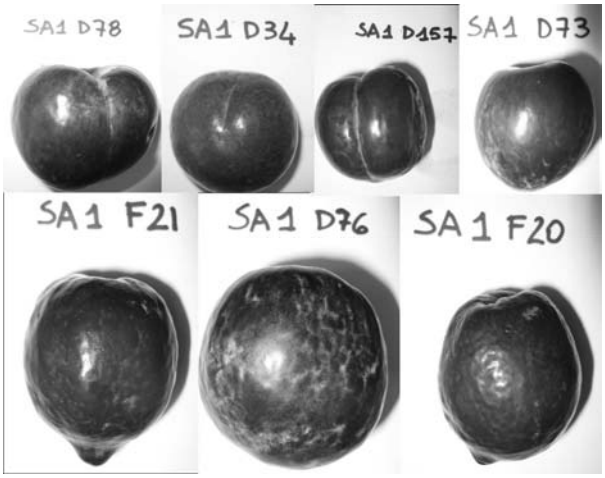

Fig. 1 Fruits from plum seedlings showing dapple fruit and rugosity symptoms

Materials and methods

Source of material: Plum seedlings (4 years old), obtained from cross breeding for quality, grown in an experimental orchard in Emilia Romagna region (Northern Italy) were used as source material. More specifically, symptomatic fruits were collected from 20 seedlings; 10 non-symptomatic fruits from seedlings located in the same orchard were also analyzed as negative control samples. HSVd-infected and healthy GF 305 were used as controls. Parental pollen donor plants ('Black sunrise' and 'Black glow'), used for breeding, were also analyzed for the presence of HSVd.

RNA target preparation and pathogen detection: For the detection of viroids (HSVd and PLMVd), total nucleic acids (TNA) were extracted from fruit skin according to the protocol established by Faggioli et al, (2001). TNA was finally eluted in 100 µL of DEPC water and analyzed following the two step/one tube RT-PCR protocol described by Ragozzino et al, (2002), using specific primer pairs (Loreti et al., 1999; Astruc et al., 1996). For the viruses detection (PPV, ACLSV, PDV, PNRSV, SLRSV, CLRV), 0.1 g of leaf sample was powdered in liquid nitrogen and total RNA was extracted using the RNeasy Plant Mini Kit (Qiagen GmbH, Hilden, Germany), according to the manufacturer's protocol. The RNA was finally eluted with 50 µL of RNase-free water and analyzed following the one-step RT-PCR protocol described by Pasquini et al. (1999), using specific primer pairs (Wetzel et al., 1991; Candresse et al., 1995; Faggioli et al., 2005; MacKenzie et al., 1997; Spiegel et al., 1997). All amplified products were analyzed using electrophoresis in a 1.5% agarose gel and stained with ethidium bromide.

Pollen isolation and extraction: Closed flowers were collected and the pollen grains mechanically separated from anthers and stored in a Petri dish. To gain evidence about the presence of the viroid inside or in the surface of the pollen grains, total RNA was extracted from 20 mg of pollen and analysed for the presence of HSVd following a modified protocol by Aparicio *et al*. (1999). Briefly, 20 mg of pollen was suspended in 900 µl of 0.2 M Tris-HCl pH 8.2, 17.5 µl of 5M NaCl, 8 µl of 10% Triton X-100 and 2 µl of 2-mercaptoethanol, vortexed for 1 min and centrifuged at 3,000 rpm for 5 min. This procedure was repeated three times. Aliquots from the three supernatants and washed homogenized pollen were phenol-extracted and the aqueous phase ethanol-precipitated and resuspended in sterile water. Supernatants and triturated pollen were analysed for the presence of HSVd by RT-PCR.

Cloning and sequence analysis: All the HSVd amplified products were purified and cloned into the pGEM®-T easy vector (Promega, Madison, WI, USA). Obtained sequences from the recombinant plasmids were multiple aligned using the Clustal W program and compared with the HSVd isolates retrieved from the Gene Bank database.

Results

Fruits from symptomatic (dapple fruit and fruit rugosity symptoms) and asymptomatic plum seedlings were analyzed by RT-PCR to test for the presence of the main viroids and viruses which affect stone fruit trees. Negative results were obtained for PLMVd and all viruses analyzed with the exception of ACLSV, which was detected rarely (data not shown). HSVd was isolated from all symptomatic samples, whereas no HSVd RNA was detected in asymptomatic seedlings. The sequencing of the cloned HSVd isolates showed a high homology (99-100 %) among the clones, with the exception of the clone F20.2 (92 %). The length of all the HSVd variants was of 296 nt (297 for the clone F20.2) and were identical to the already characterized asymptomatic plum isolates PL 278 from Turkey (Gazel et al., 2008, accession number EF523829), whereas no specific similarities were identified when comparing the HSVd plum seedlings isolates with the isolates previously found to be associated with symptoms in stone fruits.

The parental pollen donor plants 'Black sunrise' and 'Black glow' were infected with the same HSVd isolate of the fruit seedlings. The pollen collected from the parental plants also resulted positive to the test for the presence of HSVd, both inside and outside the granules.

Discussion

HSVd has been constantly found to be associated with plum seedlings showing symptoms of dapple fruit and fruit rugosity. Both these symptoms were previously associated with the HSVd infection: dapple fruit in plum and peach in Japan, Italy and China (Sano et al., 1989, Ragozzino et al., 2002; Zhou et al., 2006) and fruit rugosity in apricot in Spain (Amari et al., 2007). Our results showed that also fruit rugosity, in addition to the dapple fruit symptom, seems to be associated with HSVd in plum. The molecular characterization of the HSVd variant isolated from the symptomatic tree does not show any peculiarity. The comparison of the plum seedling isolates to the isolates previously reported in the literature in symptomatic stone fruit trees, does not highlight any typical polymorphism that could be associated with the symptoms. The symptom expression does not seem to be correlated with any peculiar nucleotide sequence of HSVd, since the most closely related HSVd isolate was an isolate identified in an asymptomatic plum in Turkey. Probably, symptom expression could be dependent on the cultivar's response.

Almost all HSVd plum seedling isolates showed a high percentage of homology (99-100 %), making it possible to hypothesize a potentially unique origin of the infection. For this reason, the parental pollen donor plants used for breeding ('Black Sunrise' and 'Black Glow') have been analyzed and found infected with the same HSVd isolate of the progeny. This result highlighted a possible role of pollen in viroid transmission. Flowers from the infected plants were collected and the pollen isolated and analyzed. The analysis performed on the pollen collected from the parental plants confirmed the hypothesis, with HSVd being found on and inside the pollen grains.

Infected pollen has been shown to play a key role in both seed and plant-to-plant transmission of several viroids, (i.e. *Potato spindle tuber viroid*- Singh et al., 1992; *Coleus blumei viroid* - Singh et al., 1991; grapevine viroids - Wah and Symons, 1997; *Avocado sunblotch viroid* - Allen et al., 1981; *Hop stunt viroid* cucumber pale fruit strain - Kryczynski et al., 1988; *Peach latent mosaic viroid* – Barba et al., 2007). The evidence of the presence of the same HSVd isolate in the parental pollen donor plants, in the pollen and in the obtained seedlings suggests a possible role of breeding in HSVd dissemination through infected seeds, even though other biological factors such as the possible transmission by thrips or other flower-working arthropods have to be taken into consideration for plant-to-plant transmission.

Studies are under way to investigate the role of infected pollen in HSVd transmission in plum, as well as to reproduce the symptomatology in healthy plum by experimental infection with the HSVd plum seedling isolate.

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