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OTHER VIRUSES

Psorosis-like Symptoms Induced by Causes Other Than *Citrus psorosis virus*

S. Martín, R. G. Milne, D. Alioto, J. Guerri, and P. Moreno

ABSTRACT. Six psorosis-like isolates from the Instituto Valenciano de Investigaciones Agrarias (IVIA) collection and two bark-scaled field sources were biologically indexed on sweet orange seedlings. Those sources were also tested for the presence of *Citrus psorosis virus* (CPsV) by ELISA, RT-PCR and EM. All induced chlorotic flecking and/or spotting in young leaves and one of the isolates (PB108) also induced psorosis B symptoms. The collection isolate RS101 and the field source PL2 did not protect against psorosis B challenge, whereas plants infected with the other sources, except PB108, showed protection. CPsV was detected by ELISA, RT-PCR and EM in PB108 and in sources protecting against psorosis B, but not in RS101 or PL2. RS101 was collected from a non-scaled adult field tree with ringspot symptoms, whereas PL2 source tree had psorosis-like bark lesions with a crater-like appearance. Three additional field sources similar to PL2 were indexed on sweet orange seedlings using green or bark lesion inoculum. They only induced young leaf symptoms, did not afford protection against psorosis B challenge, and indexed negative for CPsV by ELISA. Our results suggest that psorosis disease can be reliably diagnosed by CPsV detection. They also indicate that psorosis diagnosis based on presence of bark scaling in the field or presence of young leaf symptoms in indicator sweet orange seedlings can be misleading.

Psorosis is one of the oldest known graft-transmissible diseases of citrus (32). Field symptoms characteristic of this disease include bark scaling in the trunk and main branches, and wood staining in the lesion areas. Sometimes, chlorotic flecks, blotches or ringspots are observed in young leaves, particularly in the spring flush, but similar symptoms are also caused by other diseases such as concave gum, impietratura or cristacortis (10, 28, 29). Bark scaling usually appears when trees are 12–15 yr old, but non-scaled psorosis-infected trees aged 20 yr or more have been noted (27). This has probably favored propagation of infected buds by growers, who presumed them to be pathogen-free, thus explaining in part the high disease incidence in some areas.

Psorosis is currently diagnosed by biological indexing on different indicator plants, mainly by graft-inoculation of Pineapple sweet orange seedlings with buds or bark patches from the candidate tree and incubation in a cool (18–26°C) greenhouse (27, 28, 29). Most isolates

induce a shock reaction which causes leaf shedding and necrosis of the first flush, and then transitory chlorotic flecking and spotting in young leaves of the following flushes. The more aggressive form of the disease, called psorosis B (11, 13), additionally causes chlorotic blotching in old leaves with gummy pustules on the leaf underside and blisters on twigs (27, 28, 29). Although concave gum, impietratura and cristacortis induce young leaf symptoms similar to those caused by psorosis in the same indicator plants, specific diagnosis of psorosis can be achieved by a cross protection test using psorosis B as challenge inoculum (12, 27, 28, 29, 33). In this test, healthy sweet orange seedlings inoculated with psorosis B show the characteristic symptoms within 6 mo, whereas plants already infected with psorosis A are protected and do not show these symptoms.

The etiology of psorosis disease has not yet been demonstrated, but *Citrus psorosis virus* (CPsV), the type member of genus *Ophiovirus*

(19), has been associated with many psorosis isolates of different origins (8, 9, 14, 15, 22, 24, 25). Partial purification of CPsV allowed preparation of polyclonal and monoclonal antibodies that were used to detect CPsV by ELISA (1, 2, 6, 16, 26) and by direct tissue blot immunoassay (DTBIA) (7, 18). The CPsV genome is formed by three RNA segments of negative polarity (20, 30), which have been partially sequenced (5, 16, 31). Primers based on the available sequences were designed and used for CPsV detection by reverse transcription (RT) and PCR amplification (5, 16, 17).

A major deficiency of previous papers on detection of CPsV is that virus detection was not properly correlated with the presence or absence of psorosis disease as defined by field symptoms, biological indexing and cross protection (29). Here we have compared biological indexing and detection of CPsV by ELISA, RT-PCR and EM, in various sources of psorosis and psorosis-like diseases, differing in symptom expression. A psorosis-like bark scaling disorder was found which was not associated with CPsV infection.

MATERIALS AND METHODS

Psorosis and psorosis-like sources. The virus sources used in this study included psorosis/ring-spot isolates P121, P126, P129, PB108, P-AH and RS101 from the Instituto Valenciano de Investigaciones Agrarias (IVIA) collection in Moncada, Spain, and field sources PL1 through PL5. Isolates P121 and P129 were obtained after shoot-tip grafting *in vitro* two bark scaled Oroval Clementine trees from Chilches (Castellón). They are known to be free of tristeza, exocortis, cachexia and vein enation (22). P126 was from a severely bark-scaled Clementine tree in Moncada (Valencia). The coat protein of this isolate was smaller than others from different isolates (24). PB108

is a psorosis B isolate which has been used for many years for cross protection experiments (22, 23). P-AH was from a severely scaled navel orange propagated on sour orange in El Rocío (Huelva). Bark scaling also affected some thin branches and the trunk of the rootstock. RS101 was from a non-scaled Clementine tree showing chlorotic spots, sometimes ring-shaped, and for this reason it was included in the IVIA collection as a ringspot isolate. Field sources PL1 through PL5 were from a 60-yr-old planting of navel orange on sour orange rootstock at Vila-real (Castellón), but while the PL1 source had typical psorosis bark scaling in the trunk and main branches, sources PL2 through PL5 showed atypical bark scaling with a crater-like appearance (Fig. 1). Neither of them showed symptoms in old leaves. Some of these trees bore fruits with impietratura symptoms.

Biological indexing. Each of the above sources was graft-inoculated onto three to six Pineapple sweet orange seedlings (two bark pieces per receptor plant), grown in a potting mix of 50% sand and 50% peat moss and a standard fertilizing procedure (3). The plants were then decapitated, incubated in a temperature-controlled greenhouse (18/26°C night/day) and periodically observed for symptoms over a 6-8-mo period. Later, plants infected with each psorosis or psorosis-like source were challenge-inoculated with psorosis B isolate PB108, decapitated again, and observed during 6 additional months for psorosis B symptom onset. Controls included two healthy plants inoculated with PB108 and two more not challenged, and one plant pre-inoculated with each source and not challenged with PB108.

Lesion bark pieces from sources PL1 and PL3, PL4 and PL5 were graft-inoculated each onto two Pineapple sweet orange seedlings (two bark pieces per receptor plant) and symptoms recorded for a 6-mo period.

Fig. 1. Atypical bark scaling observed in field sources PL2 through PL5 and young leaf symptoms induced by those sources in Pineapple sweet orange seedlings.

ELISA. Serological detection of CPsV was performed by a triple antibody sandwich (TAS) ELISA as described by Alioto et al. (1). Plant extracts were prepared by trimming tissue (0.2 g) and blending it in 10 volumes of PBS buffer pH 7.4 (8 mM Na_2HPO_4 , 1.5 mM KH_2PO_4 , 2.7 mM KCl, 0.14 M NaCl) containing 0.1% Tween-20, 2% polyvinyl pyrrolidone (PVP MW 10,000, Sigma) and 2.5% defatted milk powder, using a Polytron homogenizer (Kinematica). Antiserum A322 to CPsV (16) was used for plate coating, the monoclonal antibody 13C5 (1) was used as CPsV-specific primary antibody, and rabbit anti-mouse IgG (whole molecule) immunoglobulins conjugated with alkaline phosphatase (Sigma) were used as secondary antibody. Optical density at 405 nm was measured using a Titertek Mutiscan® Plus microplate reader (Lab Systems). Samples yielding ELISA readings at least three times

the mean of the negative controls were considered positive.

RT-PCR. Detection of CPsV by RT-PCR was performed using total RNA extracts from citrus leaves and the primers CPV1 and CPV2 described by Barthe et al. (5), based on the RNA3 sequence of the CPsV-4 isolate from Florida.

Total RNA was extracted from 50 mg of young leaves with phenol/guanidine isothiocyanate (TRI-ZOL®, Life Technologies), following the manufacturer's instructions for samples with high sugar content, and RNA was resuspended in 50 μl of DEPC-treated distilled water. One-step RT-PCR was conducted in a 25- μl reaction mixture containing 1 μl of the RNA extract, 20 mM Tris-HCl, pH 8.4, 50 mM KCl, 3 mM MgCl_2 , 0.4 mM of each dNTP, 1 mM of each primer, 20 U of SuperScript II reverse transcriptase (GIBCO BRL) and 1 U of *Taq* DNA polymerase (GIBCO BRL). The thermo-

cycling conditions included 45 min at 45°C for RT, 2 min at 94°C for inactivation of reverse transcriptase and initial denaturation, 40 cycles of 15 s at 94°C, 15 s at 42°C and 30 s at 72°C, and a final elongation of 2 min at 72°C. PCR products (10 µl) were analyzed by electrophoresis in a 2% agarose gel in 1×TAE buffer (40 mM Tris-acetate pH 8.3, 1 mM EDTA) at 100 volts for 1 h. Gels were stained in 0.5 µg/ml ethidium bromide for 15 min, rinsed in water and observed in a UV-transilluminator. The 1-Kb plus ladder (Invitrogen) was used for size estimation of the PCR products.

Electron microscopy. Plant extracts were prepared by homogenizing about 6 mm² leaf tissue in 50 µl of 0.1 M phosphate buffer pH 7 containing 2% (w/v) polyvinyl pyrrolidone (PVP MW 44,000, BioRad) and were tested by ISEM. Carbon-Formvar coated grids were floated for 10–15 min on a drop of the monoclonal antibody 13C5 diluted in 0.1 M phosphate buffer, pH 7 (ascites fluid diluted 1/1000). The grids were rinsed with phosphate buffer, drained, and floated on the plant extract for a further 2 h at room temperature or at 4°C overnight.

The grids were finally rinsed plant with 30 drops of distilled water, then five drops of 1% uranyl acetate, dried, and examined in a Philips CM 10 EM at 60 KV.

RESULTS

Biological characterization.

Biological characteristics of psorosis and psorosis-like isolates from the IVIA collection and of field sources PL1 and PL2 are summarized in Table 1. While isolates P121, P126, P129, P-AH and PB108 (occasionally), and field source PL1 induced shock reactions with leaf shedding and shoot necrosis in the first flush of sweet orange seedlings, isolates RS101 and PL2 did not. All isolates induced variable chlorotic flecking and/or spotting in young leaves of the following flushes. After challenge inoculation with PB108, plants pre-inoculated with RS101 or PL2 and control plants without pre-inoculation, showed psorosis B symptoms within 6 mo, whereas the corresponding non-challenged controls and plants pre-inoculated with P121, P126, P129, P-AH or PL1, with or without challenge inoculation with PB108, remained free of those symptoms.

TABLE 1
BIOLOGICAL CHARACTERIZATION OF DIFFERENT PSOROSIS OR PSOROSIS-LIKE SOURCES AND DETECTION OF *CITRUS PSOROSIS VIRUS* (CPsV) IN THESE SOURCES

Sources	Bark scaling ^a	Biological indexing in sweet orange seedlings			CPsV detection		
		Shock	Young leaf symptoms ^b	Protect against Ps B	ELISA	RT-PCR	CPsV particles by EM
P121	+	+	+	+	+	+	+
P126	+	+	+	+	+	+	+
P129	+	+	+	+	+	+	+
P-AH	+	+	+	+	+	+	+
PB108	+	+/-	+	ND	+	+	+
RS101	—	—	+	—	—	—	—
PL1	+	+	+	+	+	ND	ND
PL2	+ ^c	—	+	—	—	—	—

^aIn field trees.

^bChlorotic flecking and/or spotting.

^cAtypical bark scaling with a crater-like aspect.

ND: Not done.

PL2 showed somewhat atypical bark scaling which also affected other trees in the same orchard. It consisted of rounded bark disruptions with a crater-like appearance that later broadened and coalesced to form irregularly shaped lesions (Fig. 1). The aspect of the bark lesion closely resembled that of psorosis affected trees. To confirm the non-psorosis nature of this bark scaling, three additional sources (PL3, PL4 and PL5) were characterized by graft-inoculation on Pineapple sweet orange seedlings using green bark or lesion bark pieces as inoculum. The source PL1 was used as psorosis control. As summarized in Table 2, plants graft-inoculated with either type of inoculum from sources PL3, PL4 or PL5 showed chlorotic flecking and/or spotting in young leaves, but not shock, and they were not protected against challenge inoculation with PB108. Contrarily, plants inoculated with PL1 green bark showed shock in the first flush, young leaf flecking and/or spotting in following flushes, and they were protected against psorosis B challenge. Plants inoculated with PL1 bark lesion showed psorosis B symptoms.

Detection of CPsV by ELISA, RT-PCR and EM. As indicated in Table 1, CPsV was detected by ELISA, RT-PCR or observation of characteristic viral particles by EM,

in plant extracts infected with P121, P126, P129, PB108, P-AH or PL1, but not in those infected with RS101 or PL2. CPsV was not detected by ELISA in plants infected with PL3, PL4 or PL5.

DISCUSSION

Our results showed that detection of CPsV by ELISA, RT-PCR or EM paralleled psorosis diagnosis based on biological indexing, including cross protection against psorosis B. The most characteristic symptom of psorosis, bark scaling, usually appears in field trees when they are 10-15 yr old, and therefore, for many years, biological indexing on sweet orange seedlings was the only diagnostic procedure to assess if non-scaled trees were psorosis-free. Since other graft-transmissible citrus diseases caused symptoms similar to those induced by psorosis in sweet orange seedlings, more specific diagnosis of psorosis required a cross protection test against psorosis B (29). However, this additional test has often been omitted, and psorosis diagnosis has been based on the presence of bark scaling in field trees or the presence of young leaf symptoms in indicator sweet orange seedlings, which can be misleading.

After the description of CPsV and its association with many psoro-

TABLE 2
BIOLOGICAL CHARACTERIZATION OF SEVERAL PSOROSIS OR PSOROSIS-LIKE FIELD SOURCES AND SEROLOGICAL DETECTION OF *CITRUS PSOROSIS VIRUS* (CPsV) BY ELISA

	PL3		PL4		PL5		PL1	
	Green bark	Lesion bark	Green bark	Lesion bark	Green bark	Lesion bark	Green bark	Lesion bark
Shock	—	—	—	—	—	—	+	+
Young ^a leaves	+	+	+	+	+	+	+	+
Ps B ^b	—	—	—	—	—	—	—	+
Cross ^c protect	—	—	—	—	—	—	+	ND
ELISA	—	—	—	—	—	—	+	+

^aChlorotic flecking and/or spotting.

^bInduces psorosis B symptoms.

^cProtects against challenge inoculation with psorosis B (PB108).

ND: Not done.

sis isolates (8, 9, 14, 15, 22, 24, 25), it has been widely assumed that it is the causal agent of psorosis. However, the etiology of this disease has not yet been demonstrated, and various agents or factors might also contribute to it. A major deficiency of many previous papers on the serological or RT-PCR detection of CPsV (1, 2, 6, 7, 16, 17, 26) was that virus detection was not properly correlated with specific diagnosis of psorosis disease based on biological indexing and cross protection (29). Our data show for the first time that CPsV detection by ELISA, RT-PCR or EM reliably reflects psorosis infection, thus extending previous results with other psorosis sources in which serological detection of CPsV was correlated with the presence of psorosis as diagnosed using the cross protection test (18).

It was interesting to observe that shock in the first flush, a symptom characteristic of most psorosis isolates, did not show up in some plants inoculated with sources carrying CPsV, which suggests that this symptom may be caused by an unevenly distributed factor (i.e. a different pathogen or a CPsV variant) or by the interaction between CPsV and certain environmental factors. Also, the isolate RS101, which was initially considered a ringspot variant of psorosis based on symptoms induced in young leaves, was shown to be unrelated to psorosis after the failure to cross protect against psorosis B, the negative CPsV ELISA and RT-PCR reactions, and the absence of CPsV particles in plant extracts. These findings confirm that none of the symptoms observed in indicator sweet orange seedlings can be reliably used to decide the presence or absence of psorosis in a candidate tree.

Atypical bark scaling observed in field sources PL2 through PL5 was initially confused with psorosis bark scaling, and failure of these sources to induce shock in indicator sweet orange seedlings was interpreted as

these sources carrying an atypical form of the disease. However, graft-inoculation of sweet orange seedlings with lesion bark from these sources did not induce psorosis B symptoms, and plants inoculated with either green bark or lesion bark did not protect against challenge inoculation with PB108, which was an indication that the syndrome observed was unrelated to psorosis. The absence of CPsV in these sources was confirmed by ELISA, and in the case of PL2 also by RT-PCR and EM. These findings indicate that diagnosis of psorosis based on the presence of bark scaling can also be misleading as has been shown for the disorder called Bahia bark scaling (4). Bark scaling observed in field sources PL2 through PL5 could be caused by a different virus or by other unknown biotic or abiotic factors. Symptoms observed in young leaves of indicator plants could be due to the presence of impietratura (symptomatic fruits were observed in some sources) or perhaps another pathogen.

Cross protection against psorosis B is considered the most reliable test for specific psorosis diagnosis (27, 29, 33), but it is expensive and lengthy (taking about 8 mo), and requires a temperature-controlled greenhouse and specialized personnel. Our results show that detection of CPsV by ELISA or RT-PCR is cheaper and faster, yet appears to yield equally reliable results. These procedures represent a major advance for easy and accurate diagnosis of different types of bark scaling, to better define the psorosis host range, or for epidemiological studies aimed to assess psorosis incidence, natural spread and potential vectors in some citrus areas. However, biological indexing on sweet orange seedlings will continue to be used in certification programs, as this is still the only way to detect other graft-transmissible citrus pathogens such as concave gum, impietratura or cristacortis (21).

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