Prunus necrotic ringspot virus Early Invasion and Its Effects on Apricot Pollen Grain Performance

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ABSTRACT

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The route of infection and the pattern of distribution of *Prunus necrotic ringspot virus* (PNRSV) in apricot pollen were studied. PNRSV was detected both within and on the surface of infected pollen grains. The virus invaded pollen during its early developmental stages, being detected in pollen mother cells. It was distributed uniformly within the cytoplasm of uni- and bicellular pollen grains and infected the generative cell. In mature pollen grains, characterized by their triangular shape, the virus was located mainly at the apertures, suggesting that PNRSV distribution follows the same pattern as the cellular components required for pollen

tube germination and cell wall tube synthesis. PNRSV also was localized inside pollen tubes, especially in the growth zone. In vitro experiments demonstrated that infection with PNRSV decreases the germination percentage of pollen grains by more than half and delays the growth of pollen tubes by ≈ 24 h. However, although PNRSV infection affected apricot pollen grain performance during germination, the presence of the virus did not completely prevent fertilization, because the infected apricot pollen tubes, once germinated, were able to reach the apricot embryo sacs, which, in the climatic conditions of southeastern Spain, mature later than in other climates. Thus, infected pollen still could play an important role in the vertical transmission of PNRSV in apricot.

Additional keywords: in situ hybridization, pollen, seed transmission.

Transmission of the virus from generation to generation in the seed takes place in $\approx 20\%$ of plant viruses (22,32); Stace-Smith and Hamilton (42) estimated that one-third of plant viruses will have been seed-transmitted in at least one host and that seed transmission has important economic consequences in at least 10 known plant virus groups. The rate of seed transmission is not necessarily a good indicator of the importance of a given disease. Some viruses are poorly transmitted by seed but, if a vector exists, the disease may be introduced into a new area. Many plant viruses are cited as being transmitted by seed but their mechanisms and routes have not been studied deeply. Based on the few studies that have been made, it seems that this mode of transmission involves infection of the embryo and can occur in two ways: directly through the mother plant or indirectly via pollen or the ovule during fertilization (24,29,46).

Prunus necrotic ringspot virus (PNRSV) causes several diseases affecting most cultivated stone fruit trees, including cherry, sour cherry, almond, peach, apricot, and plum. PNRSV belongs to the *Ilarvirus* genus and has the same genome organization as the genera *Bromovirus*, *Cucumovirus*, and *Alfavirus*, which all belong to the family *Bromoviridae*, whose encoding translation products are functionally similar (2,4,38,45). PNRSV can reduce the growth of infected trees by 30% and their yield by 20 to 56%, as well as making them very susceptible to chilling injury (44). The virus is pollen and seed transmitted (3,10,19,26), which contributes to its rapid spread in stone fruit trees (31,44). However, the mechanisms of transmission are not known in detail, mainly due to the difficulties of working with stone fruit

trees, with their long generation cycles and once-a-year blooming. Moreover, very few studies have been carried out to elucidate the route by which pollen grains become infected by plant viruses.

Infected pollen has been shown to play a key role in both seed and plant-to-plant transmission of members of the genus *Ilarvirus*. However, recent studies suggest that other biological factors, such as the mediation of thrips or other arthropods, are required for plant-to-plant transmission (17,24,30,31). Moreover, contradictory results have been obtained regarding the location of PNRSV on or inside pollen grains from infected cherry trees. Cole et al. (10) showed that PNRSV antigens were easily removed from intact pollen by washing, which suggests that most, if not all, of the virus was on the pollen surface.

Results obtained by Kelley and Cameron (26) and Digiaro et al. (11) suggest that PNRSV is located both externally and internally. Recent studies demonstrate that, in the case of nectarine (*Prunus persica*), PNRSV is located inside the pollen grain, an observation that would help to explain its vertical transmission in this stone fruit (3).

The aim of this work was to study the route of apricot pollen infection and the effects of PNRSV on pollen fitness. In addition, we will discuss the possible role of infected pollen in the vertical transmission of PNRSV in apricot trees because, in stone fruit, very little work has been done until now on how viruses invade these pollen grains.

MATERIALS AND METHODS

Plant material. Infected and healthy apricot trees (*P. armeniaca* L. cv. Búlida) from an open field at Calasparra (Province of Murcia, Spain) were checked previously for the presence of PNRSV. Flower buds, at stage 6 (8), with anthers with pollen mother cells, were collected to detect the possible presence of

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PNRSV. Flowers also were collected just before anthesis from infected and healthy trees. The flowers were divided into two groups, in one of which the presence of PNRSV was investigated in whole, mature flowers whereas, in the other, pollen grains were separated from the anthers after air drying for 24 h at room temperature.

In situ hybridization. Flower buds and anthers were fixed separately in a freshly made mixture of 4% p-formaldehyde and 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.2) at 4°C for 4 h and embedded in paraffin (Paraplast Plus; Sherwood Medical Co., St. Louis) as previously described (16). PNRSV nonradioactive riboprobe was obtained by transcribing a pPN4-890 clone, corresponding to an 890-nucleotide full-length cDNA of PNRSV subgenomic RNA 4, that codes for the coat protein (39). In situ hybridization (ISH) was performed at 55°C overnight. Slide pretreatments, hybridization, and colorigenic detection were carried out as previously described (16). Sections were examined and photographed with a LEICA DC 500 digital camera, using a DMRB LEITZ microscope (Leica Microsystems, Cambridge, UK). To study ISH with the transmission electron microscope, anthers were fixed in the same fixative as described above, post-fixed in 2% osmium tetroxide, and processed as described by Olmedilla et al. (33), with modifications. Briefly, samples were dehydrated in an alcohol series (30, 50, 70, 90, and 100%) and flat embedded in Spurr resin (41). Ultrathin sections from the same samples were picked up on copper or nickel grids, depending on whether they were going to be used for ultrastructural hybridization or ISH studies, respectively. In the case of ultrastructural studies, sections were counterstained with 5% uranyl acetate (30 min) and lead citrate (3 min) whereas, for the ISH studies, they were counterstained with 2% uranyl acetate (5 min). The probe concentration was 2 ng/μl and, as antibody, an anti-digoxigenin conjugated with 5-nm gold particles (Electron Microscopy Sciences, Fort Washington, PA) was diluted 1:30. Control experiments were carried out by performing the ISH on healthy samples processed in the same way and at the same time as infected ones.

Whole-mount ISH. Healthy and infected pollen grains were germinated in liquid medium as described by Torres et al. (43). Fixation and pretreatment of the germinated pollen grains were as described by Heuer et al. (21). Prehybridization, hybridization, and colorigenic detection were performed as in García-Castillo et al. (16), using the same probe as described above.

Dot-blot hybridization. As previously described by Aparicio et al. (3), 20 mg of pollen grains were suspended in 1.5 ml of phosphate saline-Tween polyvinylpyrrolidone buffer, pH 7.4, vigorously shaken for 1 min, and centrifuged at 3,000 rpm for 5 min. This procedure was repeated three times, followed by an additional washing with 1.5 ml of 1% sodium dodecyl sulfate (SDS) to remove particles firmly bound to the pollen grains (11). Aliquots from the four supernatants were phenol extracted and the aqueous phase was ethanol precipitated and resuspended in sterile water. Washed pollen was homogenized for total RNA extraction, as described by Sánchez-Navarro et al. (37). Supernatants and washed pollen were analyzed for the presence of PNRSV by nonradioactive dot-blot molecular hybridization (34) using the same probe as described above for ISH.

In vitro pollen grain germination. Dried pollen grains from healthy and infected trees were germinated in vitro as described previously (12,14). Each germination experiment was repeated three times (three petri dishes for each set of healthy and infected pollen grains) and three fields were chosen at random in each petri dish to count the germinated pollen grains. The average germination percentage was calculated.

In vivo pollen grain germination on pistils. Flowers from healthy trees were emasculated at anthesis and hand pollinated by either infected or healthy pollen. The pistils were removed from the flowers at 48, 72, and 96 h to study the germination of

infected and healthy pollen grains on healthy pistils. The pistils were treated and stained with aniline blue as described previously by Kho and Baër (27).

RESULTS

PNRSV detection in pollen grains. To determine the localization of PNRSV in apricot pollen grains, dot-blot hybridization of total RNA obtained from either infected or healthy pollen grains was performed, after washing them to remove possible viral particles attached to the surface and from the pollen washings. PNRSV RNA was detected in washed, infected pollen grains (Fig. 1, column 1 of infected samples) as well as in their washings. The stronger signal corresponded to the first washing with phosphate-buffered saline (PBS) (Fig. 1, column 2 of infected samples). Moreover, PNRSV RNA was detected in the SDS wash (Fig. 1, column 5 of infected samples) but not in the third washing with PBS (Fig. 1, column 4 of infected samples). These results demonstrate that PNRSV is present inside as well as on the outside of infected apricot pollen grains. On the pollen surface, PNRSV probably is attached to the exine.

It should be noted that no hybridization signal was detected from the total RNA obtained from samples of healthy pollen grains (Fig. 1, columns 1 to 5 of healthy samples).

Route of PNRSV invasion of the pollen grains. In order to study the route by which PNRSV invades pollen grains, a virus detection study was carried out in the flower buds at different development stages. The earliest stage chosen was that in which flower buds with anthers contained pollen mother cells (PMCs). At this stage, PNRSV was detected in all flower bud tissues, including sepals, petals, and anthers (Fig. 2), although not all the anthers were infected (Fig. 2B, arrowheads); others showed different degrees of infection, as can be observed at higher magnification (Fig. 2C to E). Thus, in some of them, viral RNA was localized only in the vascular tissue (Fig. 2C) whereas, in others, PNRSV had infected the vascular and connective tissues (Fig. 2D). Finally, other anthers showed the complete invasion of PNRSV, with PMCs also being infected (Fig. 2E). In all of the control experiments (use of healthy tissue), no hybridization signal was detected (Fig. 2A).

When flower buds containing unicellular pollen grains were analyzed for the presence of PNRSV, we detected the virus in all tissues of the flower bud, including the anthers (Fig. 3A). However, in this later stage, too, not all anthers or all unicellular pollen grains from each anther were infected (Fig. 3A). The PNRSV infection persisted until flower maturity, when the virus was detected in the whole flower. Moreover, in infected pollen grains in the early stages of maturation (characterized by their circular shape and still unicellular structure), PNRSV infection was uniform, with the cytoplasm, particularly, being highly infected (Fig. 3C). In none of the pollen grains studied was a PNRSV-specific signal detected in the nucleus (Fig. 3E). As regards bicellular

Fig. 1. *Prunus necrotic ringspot virus* (PNRSV) RNA detection by dot blot hybridization inside and outside infected, mature pollen grains. PNRSV was detected from infected, washed pollen grains and in their washings until 5⁻² serial dilution, the highest intensity being observed in the first phosphate-buffered saline (PBS) washing and in the sodium dodecyl sulfate (SDS) washing. Column 1: washed pollen; columns 2 to 4: PBS washings; column 5: SDS washing. There was no positive signal after using healthy pollen. Negative control: total RNA extracted from healthy cucumber. Positive control: total RNA extracted from PNRSV-infected cucumber.

pollen grains, in addition to being detected in the cytoplasm of the vegetative cell, the virus sometimes was localized around the generative cell (Fig. 3D) and sometimes inside the generative cell (Fig. 3E). No positive signal was observed when control experiments were carried out (Fig. 3B and F). The presence of viral RNA inside the generative cell was confirmed by ISH at electron microscopy level, because gold particles, corresponding to

PNRSV, were localized in the cytoplasm of the generative cell (Fig. 3G). In all of the control experiments (using healthy tissue), some gold particles also were observed by transmission electron microscopy, but only as a background signal (Fig. 3H). However, in fully mature pollen grains, which are characterized by their triangular shape, PNRSV RNA was observed, by ISH at the light-microscope level, concentrated at the apertures (Fig. 4A). This

Fig. 2. *Prunus necrotic ringspot virus* RNA localization in cross sections of young winter flowers. **A**, Healthy young flower used as a negative control for the in situ hybridization; there is no blue color in any of the structures. (Pi) pistil; (A) anthers. **B**, Infected young flower where all the tissues are highly infected (blue color), including the petals (P), except for some anthers (A and arrowheads). **C**, **D**, and **E**, Different infection levels of the anthers and the progression of infection from the filament vascular tissue (V in **C** and **D**) to the whole anther (**E**), including the pollen mother cells (PMC) and tapetum (T), are shown. Bars = 100 μ m (**A** to **D**) and 50 μ m (**E**).

Fig. 3. *Prunus necrotic ringspot virus* RNA localization in pollen grains of mature flowers. **A to F**, Light microscopy: positive hybridization is shown in blue. **G and H**, Transmission electron microscopy (TEM): positive hybridization is shown by gold particles. **A**, Cross section of a mature infected anther showing that not all the pollen grains are infected. **B**, Negative control: no positive signal is obtained by in situ hybridization (ISH) on healthy anthers. **C**, Infected unicellular pollen grain (UPG) showing the specific uniform viral RNA localization in the cytoplasm. **D and E**, Infected bicellular pollen grains (BPG) showing the specific viral RNA localization in the cytoplasm of the vegetative cell, and either **D**, surrounding the generative cell (GC) or **E**, inside the generative cell; note that there is no RNA signal in the vegetative nucleus (VN). **F**, Negative control: after ISH on healthy anthers, no positive signal is obtained. **G**, TEM of a generative cell (GC) of infected BPG shows the presence of gold particles in its cytoplasm. **H**, Ultrathin section from a healthy bicellular pollen grain showing the low background signal after ISH. Anther tissue, **A**; pollen grains, arrowheads; cell wall of the generative cell, CW; plastid, P; starch, S; vegetative cell, VC. Bars = 50 µm (**A and B**), 10 µm (**C to F**), and 100 nm (**G and H**).

observation was confirmed by whole-mount ISH (Fig. 4B). As always, no positive signal was observed when control experiments were made (Fig. 4C, healthy control for whole-mount ISH).

Effects of PNRSV on germination of infected pollen grains. When the distribution of PNRSV was studied in germinated, infected pollen grains using whole-mount ISH (Fig. 5), viral RNA was seen to be evenly distributed inside pollen grains as well as in the pollen tube, with a higher concentration at the tip or growth zone of the germinating pollen tube (Fig. 5A), whereas no signal was observed when control experiments were performed in uninfected, germinated pollen grains (Fig. 5B).

After analyzing the presence of PNRSV in infected apricot pollen grains, we studied its effect on the performance and quality of the grains. For this, healthy and infected pollen grains were germinated in petri dishes, and pollen tubes whose length was more than twice their diameter after 24 h were considered to have germinated; the germination rate in both healthy and infected pollen grains then was measured. Of the healthy pollen grains, 64% germinated, whereas only 27% of infected pollen grains did so, a reduction of more than one-half of their germination capacity. We further studied the effect of the virus on in vivo germination by pollinating healthy pistils with infected or healthy

Fig. 4. *Prunus necrotic ringspot virus* RNA localization in fully-mature pollen grains (triangular shape). **A**, Viral RNA (blue color) is specifically localized at the apertures in pollen grain sections. **B**, The same localization, at the apertures, of the viral RNA (purple color) was shown by whole-mount in situ hybridization. **C**, Negative control of the whole-mount in situ hybridization showing no purple color. Bar = 10 µm.

Fig. 5. *Prunus necrotic ringspot virus* RNA localization by whole-mount in situ hybridization in in vitro-germinated, mature pollen grains. **A**, Infected, germinated pollen grain showing the specific viral RNA (purple color) present not only inside the pollen grain but also inside the pollen tube (PT), especially at the tip. **B**, Healthy, germinated pollen grain showing no positive signal after carrying out the whole-mount in situ hybridization. Bar = 10 µm.

pollen (Fig. 6). Pollinated pistils were removed from their branches at different times (48, 72, and 96 h) to be processed and aniline blue staining was used to visualize the callose of the pollen grains and pollen tubes (see the corresponding experimental procedures section). The results show that germination of the infected pollen grains was quite poor and delayed by ≈ 24 h,

because most of them reached the middle of the healthy pistils after 72 h, whereas healthy ones did so after 48 h (Fig. 6B using healthy pollen compared with D using infected pollen, after 48 h). However, by 96 h, both infected and healthy pollen tubes had reached the corresponding ovary (data not shown), although healthy ones did so only after 72 h.

Fig. 6. Pollinated pistils, studied after 48 h and stained with aniline blue. **A**, Healthy pistil pollinated by healthy pollen, showing a high number of germinated pollen grains growing inside. **B**, Pistil area between the dashed lines in **A**, at higher magnification to show the pollen tubes germinated in the middle of the pistil. **C**, Healthy pistil pollinated by infected pollen, showing a scarce number of germinated pollen grains growing inside. **D**, Pistil area between the dashed lines in **C**, at higher magnification to show that hardly any pollen tubes are present in the middle of the pistil. Compare the two highly magnified micrographs to see the great difference in pollen tubes numbers.

DISCUSSION

PNRSV invades apricot pollen at an early developmental stage. Seed-transmitted viruses probably invade flower organs at an early developmental stage (32); more precisely, at the meristematic stage (6). Here, we have detected and analyzed the distribution of PNRSV in the floral tissues of infected apricot trees, beginning with winter flower buds that contain PMCs in their anthers. PNRSV was present in all floral organs and invaded flower buds at an early stage of development, viral RNA being detected inside PMC.

According to our results, PNRSV invades the anthers by first infecting the vascular system of the anther filament. Subsequently, PNRSV could move from the vascular system to infect the anther connective tissue, using the high number of cytoplasmic connection bridges characteristic of this tissue. Then, from the connective tissue, PNRSV invades the tapetum cells and, from there, the PMCs. Apparently, PMCs are infected before the callose layer deposition takes place, because no plasmodesmata or other cytoplasmic connections have been described in the callose layer that separates the PMCs from one another and from the anther tissue (9). Previous studies have demonstrated that transmission by seed depends on early infection of the PMCs, as in the case of *Lettuce mosaic virus* (23) and in the seed-transmitted isolate of *Barley stripe mosaic virus* (9).

It seems reasonable to think that isolation of the PMCs (which are directly involved in sexual plant reproduction) from other anther tissues, which takes place in nature to separate the gametophyte and the sporophyte generations, could have developed in plants as a natural resistance mechanism to prevent pathogen transmission from the parents to the new generation, producing virus-free pollen grains.

The location of PNRSV in apricot pollen grains might explain its vertical transmission. Previous results have shown that PNRSV is localized on the surface of infected pollen grains (10,26). In nectarine, it was located mainly inside infected pollen grains (3) whereas, in apricot, we detected PNRSV particles both inside and on the surface of infected pollen grains using different experimental methods. Taking our data and those from other studies together, it seems that the internal or external localization of PNRSV in the pollen grains depends on the particular *Prunus* sp.

We observed a uniform distribution of viral RNA in the cytoplasm of unicellular and bicellular infected pollen grains and in the generative cell of the latter. Using ISH, Aparicio et al. (3) detected PNRSV RNA in the cytoplasm of unicellular and bicellular infected nectarine pollen grains, although only in the vegetative cytoplasm, not in the generative cell, which is the cell really involved in the fertilization process. Based on our data and those from previous studies (10,26), it seems that PNRSV can be transmitted to embryos during fertilization, either from the infected generative cell or from the cytoplasm of the vegetative cell. The latter view is supported by previous results (36), which show that some cytoplasm of the vegetative cell reaches the newly formed zygote through the pollen tube during fertilization.

In our work, ISH detected PNRSV in fully mature, infected apricot pollen grains, mainly in the apertures. Although Kelley and Cameron (26) reported the same localization for PDV particles in infected cherry pollen grains, they observed that this localization might be an artefact due to the use of potassium permanganate as a fixation agent. In our case, the fixation agent used was that described by Karnovsky (25), characterized by its rapid penetration and good preservation of cell contents. Moreover, our studies of pollen grains under the electron microscope showed that the ultrastructure was well preserved (data not shown). Taking into account all these data, we can confirm that the localization of PNRSV at the apertures of infected pollen grains is not an artefact. Torres et al. (43) detected hydroxy-

proline-rich glycoprotein, which is an abundant component of the pollen tube cell wall, in the apertures and in the growth zone of the pollen tube. This localization is similar to that of PNRSV in our infected apricot pollen grains and along the pollen tube, with a high accumulation in the growing zone. Thus, PNRSV might be following the same distribution pattern as the cytoplasmic components involved in the synthesis of the pollen tube wall. This cytoplasmic localization of PNRSV at the pollen grain apertures could be the mechanism used by viruses for transmission to embryos through the cytoplasm of the vegetative cell (which was highly infected in the apricot pollen grains).

PNRSV affects apricot pollen germination but not its possible participation in the fecundation process. The quality of pollen, estimated by its germination rate, directly affects fruit set (47). Benediková (5) demonstrated a positive correlation between fruit production and the germination rate of some apricot cultivars. In our study, PNRSV was seen to affect the pollen germination capacity, which it reduced by $\approx 58\%$ in infected apricot pollen. This effect on pollen germination also has been observed in other virus–host combinations (28,48). However, a negative effect of a virus on pollen quality is not a limiting factor for seed transmission through infected pollen (15,20,32).

Moreover, infected apricot pollen grains were able to germinate in vitro and inside pistils, although the growth of the pollen tubes was delayed by ≈ 24 h. This effect also has been observed in other virus–host combinations (24). Normally, such a delay would be a limiting factor for fertilization by infected pollen, due to competition with healthy pollen, because not all the apricot pollen grains from the same infected flower, or all the apricot flowers from the same infected tree, were infected. Moreover, in the weather conditions of Mediterranean areas and especially in southeastern Spain, where this work was carried out, this competition would not be sufficient to prevent PNRSV transmission by infected apricot pollen. Although both healthy and infected pollen grains are mature at anthesis, some apricot embryo sacs from many cultivars, including Búlida used in this work, mature ≈ 4 to 6 days later than in other climates (1,7,13,18). This delay in embryo sac maturation could be an advantage for the slower-growing PNRSV-infected apricot pollen grains because, even if healthy pollen reaches the base of the ovary, it will not be attracted by the apricot ovule if the apricot embryo sac is not yet fully mature (35,40).

In short, our studies have shown clearly that PNRSV invades apricot reproductive tissues at a very early developmental stage, infecting PMC. However, although infection affected apricot pollen grain performance during germination, PNRSV did not completely prevent fertilization, because the infected apricot pollen tubes, once germinated, were able to reach the apricot embryo sacs which, in the climatic conditions of southeastern Spain, mature later than in other climates. Thus, infected pollen still could play an important role in PNRSV vertical transmission in apricot.

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