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A model system for plant-virus interaction—infectivity and seed transmission of *Cherry leaf roll virus* (CLRV) in *Arabidopsis thaliana*

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Abstract The wide natural incidence of *Cherry leaf roll virus* (CLRV) in deciduous forest trees and nurseries in northern Europe is believed to have occurred, apart from occasional mechanical spread and transmission through grafting, mainly by seed transmission. The mode of the vertical transmission and its role in the epidemiology of the virus has not been investigated, basically due to the inconvenient host-pathogen combinations studied to date. With the aim of obtaining an appropriate system for identification of viral genes and products participating in infection processes and seed transmission of CLRV, we performed infection and seed transmissibility tests with CLRV in *Arabidopsis thaliana* plants. Two phylogenetically and serologically different CLRV isolates were tested. Both of them were found able to infect *A. thaliana* plants, exhibited clear symptoms of the infection and spread systemically in the plants. Infection of the seeds and of a remarkable number of seedlings generated from infected seeds was possible for two consecutive generations. These results, for first time, report seed transmission of CLRV in the model plant *A. thaliana* and allow the assumption to be made of embryo invasion during seed transmission. Furthermore, first indications are given that genetically diverse CLRV isolates exhibit different abilities for vertical transmission in *A. thaliana*. The CLRV-*A. thaliana* model system is suitable for investigating viral invasion of developing plant organs and meristematic tissue, a prerequisite for successful virus dissemination via vertical transmission through seed.

Keywords Nepovirus · Virus infectivity · Vertical transmission · Model system

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Cherry leaf roll virus (CLRV) (genus Nepovirus, family Comoviridae) is of particular scientific interest, as it is globally distributed in a wide range of herbaceous and woody plants (Bandte and Büttner 2001; Cooper and Atkinson 1975; Jalkanen et al. 2007; Jones 1986). The most common natural hosts of CLRV are common birch (*Betula pendula*), black elderberry (*Sambucus nigra*), English walnut (*Juglans regia*) and sweet cherry (*Prunus avium*). Transmission of CLRV by mechanical inoculation has been confirmed in numerous indicator plants from the

Chenopodiaceae, Compositae and Solanaceae families (Baumgartenerova and Slovaka 1995; Henriques 1994). Transmission by vectors has been poorly reported and, unlike most members of the genus *Nepovirus*, CLRV is not considered to be transmitted by soil-borne nematodes (Cooper and Edwards 1980; Jones et al. 1981; Wang et al. 2002). Recently, it has been suggested that CLRV particles released from roots of infected *Chenopodium quinoa* plants were transferred through nutrient solution and infected healthy *C. quinoa* plants (Bandte et al. 2007; Laza 2007, unpublished data).

Vertical transmission of CLRV by seed and pollen has been reported to occur naturally (Bandte and Büttner 2001; Dosba et al. 1990; Jones 1986). Seed transmission is an essential property of at least 25 virus genera (Mink 1993), covering approximately 18% of all plant viruses (Maule and Wang 1996) including 19 reported species within the genus *Nepovirus* (Rubies-Autonell and Turina 1997) and represents a natural pathway through which virions are transmitted to the progeny of the hosts and spread to the environment. Due to pollination barriers, vertical transmission of viruses limits interspecific viral transmission; the female pistil presents an elaborate barrier that shields ovules from access to pollen from other species (Swanson et al. 2004). However, seed transmission is supported by other methods of diffusion (e.g. vectors carrying seeds), increasing the efficiency of virus dissemination in nature (Mink 1993). Moreover, seed transmission threatens gene banks with contamination; through seed, a virus may enter a mother plantation without detection and, through the propagative material, may follow a broad, human-mediated propagation and dispersal route. Due to this property, CLRV is included in the list of plant viruses that should be closely monitored during sanitary production of propagative material, especially for walnut and olive trees (Bassi and Martelli 2000).

Arabidopsis thaliana was chosen as a host of CLRV to investigate mechanisms of seed transmission. CLRV hosts studied to date were either woody plants with a long-life cycle, which additionally impedes virus detection due to irregular distribution or low virus concentration (Büttner and Bandte 2002), or herbaceous indicator plants with limited availability to their genomic data. *Arabidopsis thaliana* is appropriate for such a study because of its short life-cycle and the production of a large number of seeds. The genome of *A. thaliana* is well characterised (Meinke et al. 1998), and data on protein profiles and the functions of this model plant are easily determined (Xi et al. 2006), facilitating the investigation of interactions of viral components with plant factors (von Bargen et al. 2001). Moreover, it has been used previously as a host plant for different plant viruses such as *Tomato spotted wilt virus* (TSWV, German et al. 1995), *Tobacco mosaic virus* (TMV) and *Turnip yellow mosaic virus* (TYMV), including studies of virus seed transmission (de Assis Filho and Sherwood 2000). As CLRV can be readily transmitted by mechanical inoculation to a wide range of herbaceous species, it was assumed that it could also successfully infect *A. thaliana*.

Arabidopsis thaliana ecotype Columbia (Col-0) seedlings were cultivated in propagation substrate (generation F0). After 4 weeks, four rosette leaves of each plant were mechanically inoculated using fresh leaf homogenate from the CLRV-infected propagation host *Nicotiana clevelandii* prepared in 0.01 M sodium phosphate buffer. Inoculated plants were further cultivated under greenhouse conditions (temperature: 21–23°C, RH: 40–60%, 16 h daylight). Twelve *A. thaliana* plants were inoculated with a CLRV-E395 isolate originating from rhubarb (*Rheum rhabarbarum*) (group A). A second group of 23 *A. thaliana* plants (group B) were inoculated with isolate CLRV-E603 originating from black elderberry (*S. nigra*) (Rebenstorf et al. 2006). Three mock-inoculated *A. thaliana* plants served as negative controls.

Symptom development of inoculated plants was monitored and CLRV infection determined by virus detection through Immunocapture Reverse Transcription-PCR amplification (IC-RT-PCR) with CLRV-specific primers RW1 and RW2 (Werner et al. 1997) in inoculated leaves and/or blossoms. CLRV isolates were discriminated by Restriction Fragment Length Polymorphism (RFLP) analysis of PCR products with the restriction endonuclease EcoRI to cleave the 412 bp fragments generated by PCR. Amplicons from plants infected with the elderberry isolate (CLRV-E395) produced two bands of 231 bp and 181 bp after EcoRI digestion, while IC-RT-PCR products originating from CLRV-E603-infected plants remained uncut (Fig. 1).

Four out of 12 *A. thaliana* F0 plants inoculated with the CLRV-E395 isolate developed symptoms. Inoculated leaves showed interveinal chlorosis 15–

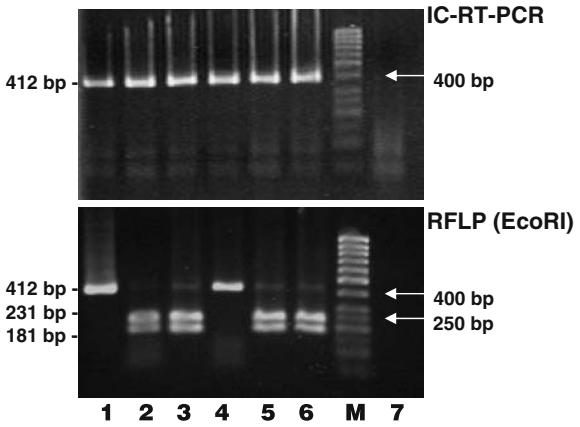

Fig. 1 CLRV detection by IC-RT-PCR in inoculated *A. thaliana* leaf samples (*above*) followed by differentiation of isolates CLRV-E395 and CLRV-E603 by RFLP analysis of the 412 bp amplification products (below). Lines 1 and 4 = plants inoculated with virus isolate CLRV-E603; lines 2, 3, 5, and 6 = plants inoculated with CLRV-E395; line 7 = mock-inoculated plant; *M*=50 bp Ladder, Fermentas

17 days post-inoculation (dpi) and rapidly died, while the new leaves became systemically infected as shown by the appearance of peripheral chlorosis, irregular purpling and leaf roll (Fig. 2a). Of the 23 *A. thaliana* plants inoculated with isolate CLRV-E603, 22 plants developed similar symptoms as plants inoculated with isolate CLRV-E395; however, the malformations in inoculated leaves appeared earlier and more severe (8–10 dpi, Fig. 2b).

IC-RT-PCR testing of inoculated leaves from the 12 CLRV-E395-inoculated plants 23 dpi resulted in five infected plants, four plants showing symptoms and one without visible features of a virus infection. Seven plants did not develop symptoms and were negative in the IC-RT-PCR tests. Systemic viral spread was confirmed in the four CLRV-E395-inoculated plants that expressed symptoms (A4, A5, A8, A12) by subjecting inflorescences (35 dpi) to IC-RT-PCR; only part of the inflorescences was removed allowing the rest to bloom and form seeds. From these plants seeds were collected and plant lines (five F1 seedlings/F0 plant) were generated for further seed transmissibility tests (Fig. 3). Testing of leaf samples or/and inflorescences from 19 CLRV-E603-inoculated plants resulted in 16 infected plants at sampling time 15 dpi. Two symptomatic plants as well as the one asymptomatic plant tested were negative in IC-RT-PCR. Systemic spread of viral isolate CLRV-E603 in *A. thaliana* was shown in three symptomatic F0 plants (B5, B6, B17) by probing inflorescences 4 weeks after inoculation. An inflorescence from plant B1 was not tested, though a seed sample (approx. 100) collected from this plant gave positive results. Seeds were collected from these four plants and plant lines with five plants each were included in further seed transmission studies (Fig. 3).

In the F1 generation derived from CLRV-E395-infected plants (group A), in all seed samples (approx. 100 seeds/sample) CLRV was detectable by IC-RT-PCR. However, the presence of viruses in seeds does not always lead to seedling infection; a virus may be either seed-borne, being carried by the seed without infecting the seedling, or seed-transmitted, infecting the seedling produced by the seed (Agarwal and Sinclair 1996). To test for seed transmissibility of CLRV, from the four seed lots 20 seedlings were sown and whole seedlings were subjected to IC-RT-PCR 58 days after sowing (das). Thirteen seedlings out of 17 tested were found to be CLRV-infected (76.5% CLRV detection rate). At least two CLRV-

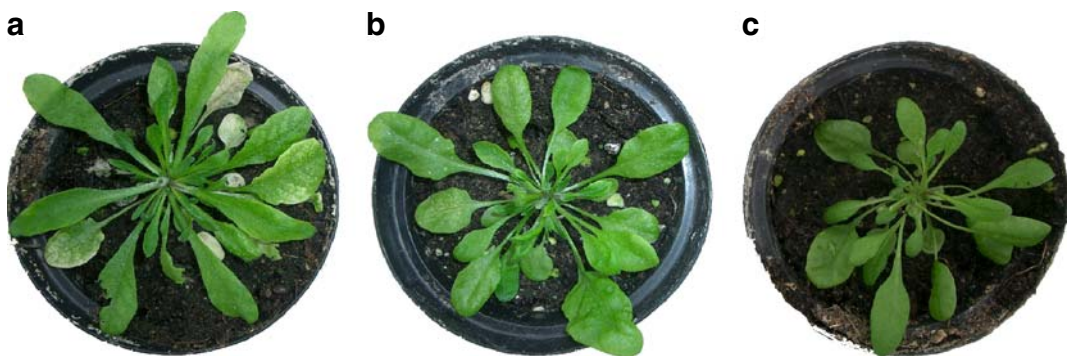

Fig. 2 F0 generation *Arabidopsis thaliana* Col-0 plants **a** infected by CLRV-E395 isolate (19 dpi), **b** infected by CLRV-E603 isolate (10 dpi) and **c** negative control (10 dpi)

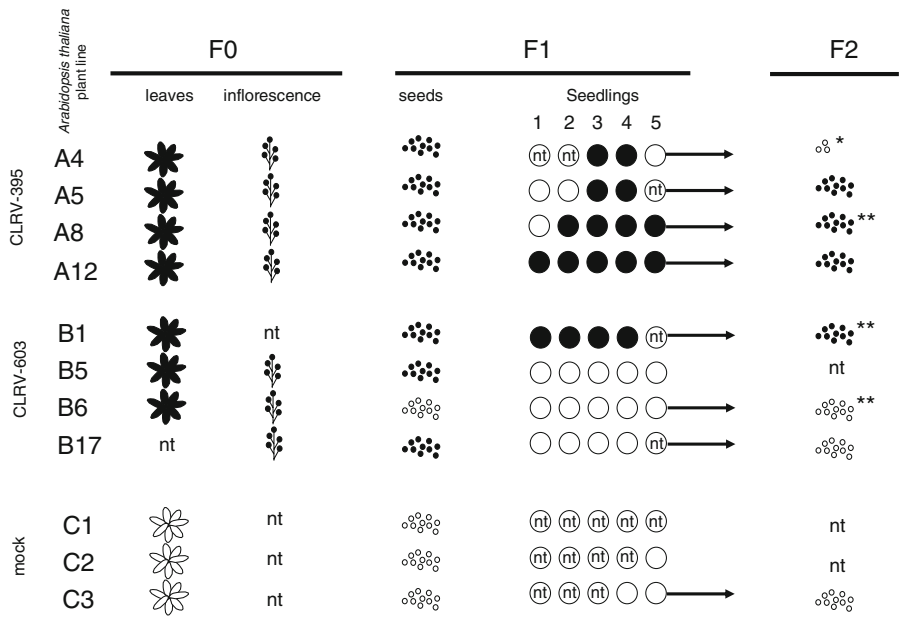

Fig. 3 CLRV detection in three consecutive generations of *A. thaliana* plant lines *black* = CLRV-infected, *white* = healthy, *nt* = not tested, *<30 seeds, **<100 seeds. The *arrows* indicate F1 plant of investigated F2 generation seed lots

infected seedlings originated from every seed lot, which proved virus transmission in *A. thaliana* to the next generation by seed. The F1 generation originating from four CLRV-E603-infected plants revealed three infected seed lots out of four but few infected seedlings. Out of 15 F1 plants tested 58 das, only four seedlings (26.7% CLRV detection rate) all originating from the same parent (B1) were CLRV-positive in IC-RT-PCR. By comparison of the detection rates of tested F1 seedlings with the two virus inoculation variants, it can be assumed that seed transmission was more successful using isolate CLRV-E395 than CLRV-E603. However, the real seed transmission rate may be higher than the one calculated by the given data, because not every seedling was included in IC-RT-PCR.

Testing of F2 generation seeds again revealed higher virus contamination of seed lots derived from CLRV-E395-infected plant lines than pooled seeds collected from F1 generation plants of group B (CLRV-E603 plant lines) (Fig. 3; column F2).

Three mock-inoculated controls (C1, C2, C3) did not develop any symptoms and gave consistently negative IC-RT-PCR results in the F0 generation. Three lines of mock plants were sown and the tested samples at 58 das as well as the seed samples of the F2 generation were negative (Fig. 3; mock).

Summarising the infectivity and seed transmissibility tests, the success and rate of seedling infection characterised the CLRV presence in the *A. thaliana* lines and revealed three trends. First, lines from plants A12 and B1 can be described as 'black' lines (Fig. 3), because from the F0 to F2 generation all tested samples were positive for CLRV. Second, lines from A4, A5 and A8 plants were, 'mostly black', meaning that all samples were positive but for a few F1 seedlings which were negative. Finally, B5 and B17 lines were 'half-black', meaning that they were positive until the F1 stage of seeds but then turned negative. In the line derived from the B6 plant, only F0 generation samples were infected. The mock plants can be characterised as 'white' lines, as samples tested from F0 to F2 generations were negative.

Diverse causes may lead to the failure of seedling infection when virus is present in the seed: the virus may not be infectious anymore, the virus titer may be too low, virions may be damaged due to physiological modifications during seed maturation or the virus may be in a part of the seed that hampers further infection (Johansen et al. 1994). Seed transmission is most often linked to embryo invasion, while virus presence in the seed coat is insufficient to cause seedling infection (Agarwal and Sinclair 1996; Maule and Wang 1996). Therefore, results presented here on infected seedlings produced by infected seeds suggest virus presence in the embryo. However, studies on *A. thaliana* seed transmission of TYMV and TMV demonstrated that embryo invasion was necessary but not sufficient to lead to seed transmission (de Assis Filho and Sherwood 2000).

The two CLRV isolates used in the study were capable of infecting *A. thaliana* and of being transmitted through seed but showed differences in infectivity, symptom severity and seed transmissibility. The rhubarb CLRV-E395 isolate infected 41.7% of inoculated plants five out of the 12), facilitated a long survival of inoculated leaves and produced milder systemic symptoms but had a higher incidence of seed transmission. The black elderberry CLRV-E603 isolate led to higher infection rates (14 out of 17 of inoculated plants; 82.4%), symptoms appeared one week earlier than in the inoculations with the CLRV-E395 isolate and caused death to all rosette leaves, but usually failed to be transmitted through seed. The two isolates belong to different phylogenetic clusters and MAb reactivity groups (E603: group E and 4; E395: group B and 2, respectively). CLRV-E395 belongs to a phylogenetic cluster which comprises isolates originating from a wide range of host plants (ash, rhubarb, ground elder), while CLRV-E603 is a member of a cluster with isolates originating mostly from black elderberry with few exceptions (Rebenstorf et al. 2006). In contrast to the expectation that high virulence characterises isolates with a wide host range, the black elderberry isolate showed high infectivity in *A. thaliana*.

In conlusion, the *A. thaliana*-CLRV system is a suitable model for studying plant-pathogen interactions during infection and seed transmission. By comparative studies using CLRV isolates exhibiting differences in seed transmission in this model plant, more knowledge about the molecular interaction of plant and pathogen may be gained. Remarkable also is the fact that high infectivity after mechanical inoculation occurred in parallel to low vertical transmission of CLRV in this virus-host plant system. The detection rate of CLRV in seed was higher than that in seedlings generated from infected seeds, which agrees with observations from other transmission studies in various virus-plant combinations (Mink 1993; Rubies-Autonell and Turina 1997; de Assis Filho and Sherwood 2000; Müller et al. 2006). Consequently, CLRV detection in seed is not an appropriate indicator of seed transmission. Detection only in germinated progeny is clear evidence of vertical transmissibility of the virus. Further studies are required to confirm virus invasion of the floral meristem and the virus localisation in the gametophytes and gametes, which would consequently cause embryo invasion and subsequently seed transmission.

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