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SHORT COMMUNICATION

Identification of a *Lotus* viral pathogen

O. Schumpp · M. E. Ramel · P. Gugerli · W. J. Broughton · W. J. Deakin

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Abstract A virus collection was used to identify a pathogen suitable for laboratory use with the model legume *Lotus japonicus*. Several *Lotus* species or *L. japonicus* accessions were tested and various degrees of susceptibility to the *Arabis mosaic virus* derived from barley (ArMV-ba) were found. Virus multiplication and persistence in *Lotus* tissue were examined, as well as plant responses to it. Sensitivity to the virus among the accessions and species is discussed in light of their geographical origin.

Keywords *Arabis mosaic virus* · Plant defence · Virus-induced gene silencing

Lotus japonicus is used as a model legume in the study of symbiotic plant–microbe interactions with rhizobia, and numerous *Lotus* genes important for the symbiosis have been identified (Udvardi et al. 2005). We are interested in the mechanisms by which *Lotus* perceives the variety of micro-symbionts that are able to induce nodule formation, and particularly why some rhizobia are inefficient micro-symbionts (Banba et al. 2001). One possibility could be that *Lotus* recognises inefficient symbionts as potential pathogens and mounts a defence reaction. Thus we set out to identify marker genes specifically associated with defence responses in *Lotus*. This is complicated by the fact that, although bacterial diseases of *Lotus* have been reported in the field (Alippi 2005), no microbial pathogen is available for laboratory studies. We screened a selection of viral isolates, based on their broad host-range or their ability to develop disease on taxonomically related legumes (Table 1), from the plant virus collection of the Agroscope Changins–Wädenswil Research Station, Switzerland. Viruses were chosen because monitoring viral infection of plants is greatly facilitated by enzyme-linked immuno-sorbent assays (ELISA). Furthermore, plant defence responses to viruses recruit R-gene-dependent signalling pathways that are also involved in general defence against non-viral microbes (Kachroo et al. 2006).

Viral stocks were reactivated on their most appropriate host to ensure that large numbers of fully infectious viruses were prepared; infected leaves were then ground in cold phosphate buffer and this extract used to mechanically inoculate legumes. Initial screening was performed on *L. japonicus* Gifu B-129, *L. corniculatus* and *Trifolium pratense* L. var. *pratense*. Four weeks after inoculation, individual plants were assayed for viral spread. Surprisingly, both *Lotus* species were resistant to almost all the viruses known to infect legumes such as *Medicago sativa* (alfalfa) or *Trifolium pratense* (red clover). Only *Tobacco ringspot virus* (TRSV) and *Arabis mosaic virus* from barley (ArMV-ba)—isolate RAC 1087—were able to occasionally infect *Lotus*. A large-scale trial was initiated, ArMV-ba was reactivated on a susceptible host, *Chenopodium quinoa*, and 40 *L. japonicus* Gifu B—129 plants were mechanically inoculated. ArMV-ba was detectable in one plant, first in its roots and later in all parts of the shoot. This plant was also only temporarily infected however, as controls performed 8 weeks after inoculation were

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O. Schumpp · W. J. Broughton (✉) · W. J. Deakin
LBMPS, Sciences III, Université de Genève,
1211 Genève 4, Switzerland
e-mail: William.Broughton@bioveg.unige.ch

M. E. Ramel · P. Gugerli
Virology Department, Agroscope Changins–Wädenswil,
1260 Nyon 1, Switzerland

Table 1 Screening of various legumes for sensitivity (+ or –) to different viruses. Infections were assayed by enzyme-linked immuno-sorbent assay (ELISA)

Plant virus	Acronym	Isolate number	*Lotus japonicus* Gifu B–129	*Lotus corniculatus* L.	*Trifolium pratense* L. var. *pratense*
Alfalfa mosaic virus	AlMV	RAC 1024	–	–	+
Alfalfa mosaic virus	AlMV	RAC 1034	–	–	+
Arabis mosaic virus	ArMV	RAC 1087	+	nt	nt
Clover unidentified virus[a]	–	RAC 799	–	–	+
Clover unidentified virus[b]	–	RAC 907	–	–	+
Cucumber mosaic virus	CMV	RAC 883	–	–	+
Red clover necrotic mosaic virus	RCNMV	PV 0018[c]	–	nt	nt
Tobacco rattle virus	TRV	RAC 1223	–	nt	nt
Tobacco ringspot virus	TRSV	RAC 1081	nt	+	–
Watermelon mosaic virus 2	WMMV-2	RAC 1082	–	nt	nt

nt Not tested

[a] Mixture of unidentified potexvirus and closterovirus

[b] Mixture of unidentified potexvirus and isometric virus

[c] Isolate from the German collection of microorganisms and cell cultures (*DSMZ* Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH)

negative. ArMV-ba was isolated from this plant after 4 weeks and used to infect four more Gifu plants; one plant became stably infected as the virus was still detected throughout the plant after 3 months. It appeared that using ArMV-ba propagated in Gifu plants as inoculum increased subsequent *Lotus* susceptibility. In all further experiments inocula were prepared from fresh tissues of (ELISA-confirmed) infected Gifu plants. Nine *L. japonicus* accessions and two other *Lotus* species were assayed for their susceptibility to Gifu-derived ArMV-ba by ELISA detection of viral presence. In these experiments 70% of the plants from certain accessions were infected by the virus, whereas other accessions were fully resistant (Fig. 1). The two most susceptible (MG-20 and MG-52) and the initially most resistant accession (MG-23) were re-tested with larger numbers of plants (97, 60, and 59, respectively) with plants becoming infected to levels observed previously (Fig. 1).

ArMV-ba was initially identified on barley by Gugerli and Ramel (1996) and is one of the rare nepoviruses that are able to infect cereals. Although it is antigenically closely related to grapevine ArMV isolates, it produces slightly different symptoms (Gugerli 2004). ArMV isolates normally have a large host-range, with some isolates being able to infect 93 different plant species from a test population of 138 species (Schmelzer 1962). ArMV isolates are predominantly associated with dicotyledonous species. In

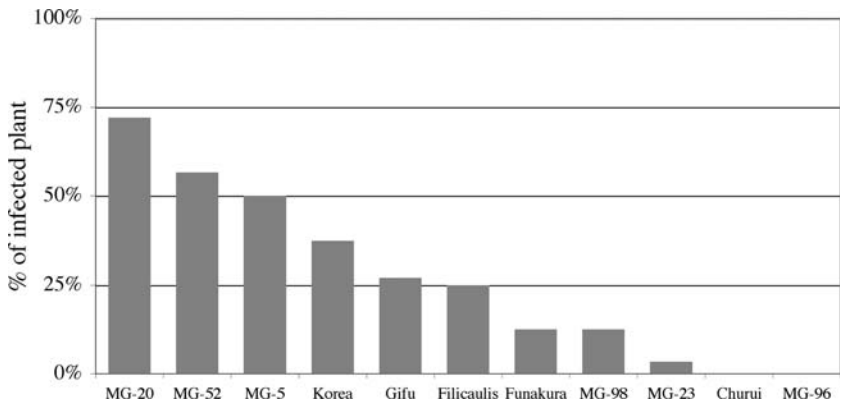

Fig. 1 Plants were sown in soil and grown with natural light at 20 ± 2°C for 4–5 weeks before inoculation with virus. Mechanical inoculation was performed with tissues ground in 20 mM phosphate buffer pH 7.6 containing 10 mM sodium diethyldithiocarbamate. Infection was assayed by enzyme-linked immuno-sorbent assay (ELISA) with a polyclonal *Arabis mosaic virus* (ArMV) antibody from BIOREBA (Reinach, Switzerland). Infection experiments were first performed on eight plants of each ecotype. Gifu B-129, MG23, MG52 and MG-20 were double checked with 40, 59, 60, and 97 plants, respectively

most cases the symptoms are mild discolorations that often disappear soon after infection, but dramatic effects on plant development have been observed on a few species. With *Lotus*, a weak mosaic pattern was occasionally found on the most susceptible species about 10 days after inoculation (Fig. 2), but this disappeared within a few days leaving inoculated plants indistinguishable from non-inoculated controls.

We checked for viral spread, persistence and the appearance of any potential symptoms during *Lotus* infection. When ArMV-ba was detectable in a plant, within 7 days the virus had spread throughout the roots and shoots, as shown by sampling various parts of the plant distant from the inoculation site (data not shown). In a time-course experiment with 40 inoculated Gifu plants, weekly assays showed that the number of plants remaining infected was stable (Fig. S1). The virus persisted over long periods; *Lotus* accessions still contained the virus 2–3 months after inoculation. Despite the lack of any obvious symptoms or effects on plant growth, seeds from infected plants had a reduced germination efficiency, as has been previously observed with infected barley (Gugerli 2004). Neither qRT-PCR with putative molecular markers for defence-related genes, such as chitinase, chalcone synthase and phenylalanine ammonia lyase, nor a PCR-based differential display technique revealed any differences in transcription of Lotus genes (data not shown).

Fig. 2 Symptoms visible as a mild mosaic that appeared 10–15 days after inoculation. The *Lotus* accession shown is MG52 and the presence of *Arabis mosaic virus* from barley (ArMV-ba) was confirmed by enzyme-linked immuno-sorbent assay (ELISA)

Interestingly, we noticed that susceptibility of *L. japonicus* accessions and related *Lotus* species correlates with their geographical distribution (Fig. 3). Species such as Churui and MG96, which are found in the northern-most latitudes, were fully resistant, whereas more sensitive species were found in the centre and the south of the Japanese archipelago. The most sensitive species, MG-20, originates from Okinawa (Kawaguchi 2000), approximately 2,000 km south of the most resistant one, MG-96. This observation suggests that there has been a dissemination of resistance during evolution of these varieties. Two distantly related species, *Lotus korea* from Korea (Jiang and Gresshoff 1997)

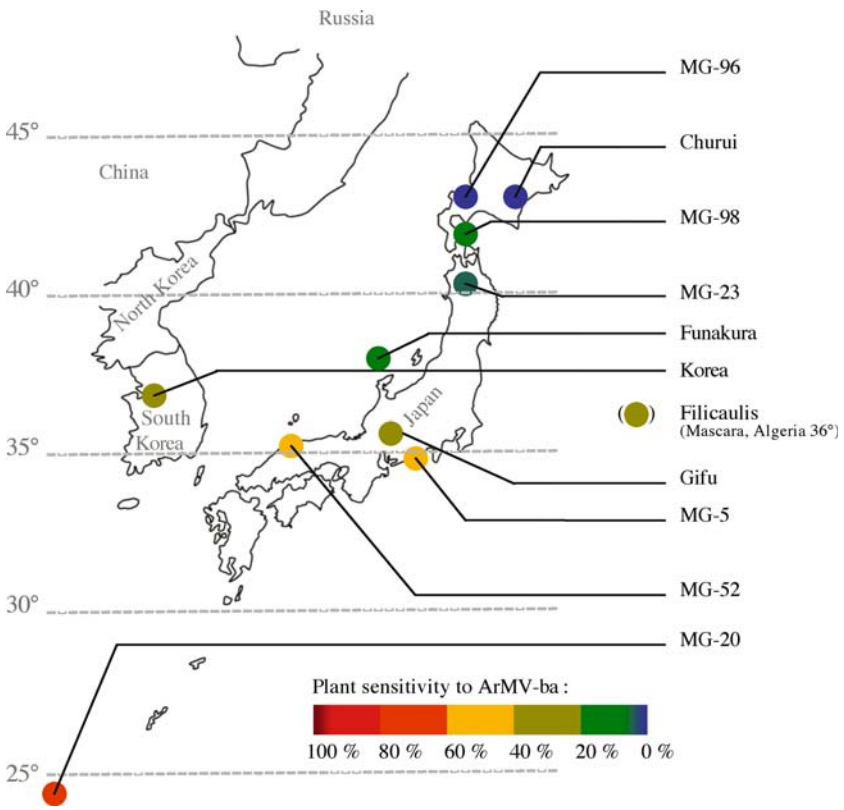

Fig. 3 Geographical origin of *Lotus* spp. or *Lotus japonicus* accessions susceptible to ArMV-ba. Infection was assayed by enzyme-linked immuno-sorbent assay (ELISA). The origin of *Lotus filicaulis* is described in Durieu de Maisonneuve (1846)

and *L. filicaulis* (Durieu de Maisonneuve 1846) from Algeria, have virus susceptibilities very similar to *Lotus japonicus* accessions growing at similar latitudes in Japan. The degree of virus susceptibility thus cannot be explained by adaptive radiation of the resistance, as the geographical separation between the *Lotus* species is too large. We think it more likely that levels of *Lotus* resistance to mechanical inoculation of the virus are related to plant traits associated with day length or duration of the growing season, which have evolved to environmental conditions at the latitudes where the plants grow naturally.

In conclusion, we have identified a pathogen of *Lotus* that can be mechanically transmitted. Following inoculation with ArMV-ba, *Lotus* plants exhibit only minor reactions. No long-lasting symptoms are apparent, nor is there any effect on flowering or growth, as is the case with other plants. The absence of symptoms was not due to loss of the virus however, as in most cases virus particles could be detected in infected *Lotus* throughout the experiment. ArMV-ba can disperse throughout the shoots and roots (where it can remain for several weeks without generating persistent visible symptoms) of important *Lotus* species or accessions widely used in the scientific community such as Gifu, MG-20 and *L. filicaulis*. As with all nepoviruses, the ArMV-ba genome consists of two positive-sense single-stranded RNAs (Mayo and Robinson 1996), and is similar to the genome of *Pea early browning virus*, a tobravirus transmissible by nematodes that has already been successfully transformed into an infectious vector adapted for targeted virus-induced silencing (VIGS) in pea (Constantin et al. 2004). All these characteristics make ArMV-ba a good candidate for VIGS in *Lotus*. We are currently developing an ArMV-based vector suitable for VIGS in *L. japonicus*.

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