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Virus-vector relationships of chickpea chlorotic dwarf geminivirus and the leafhopper *Orosius orientalis* (Hemiptera: Cicadellidae)[1]

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Summary

Chickpea chlorotic dwarf geminivirus (CCDV) is one of the viruses associated with chickpea stunt disease. It is transmitted by the leafhopper *Orosius orientalis*. The minimum acquisition access period (AAP_{min}) and inoculation access period (IAP_{min}) were found to be less than 2 min, while the minimum latency period (LP_{min}) was less than 2 h. The median AAP, IAP and LP were 8.0 h, 2.3 h and 27.7 h, respectively. No difference in transmission rates (proportion of leafhoppers able to transmit) was observed between male and female leafhoppers. In serial transmission experiments, transmission was shown to be persistent, and after a 2-day AAP about 80% of the leafhoppers transmitted the virus for most of their life. The virus could be detected in individual leafhoppers by DAS-ELISA. It did not multiply in the leafhopper, but, instead, decreased in concentration during leafhopper feeding on a non-host of the virus.

Key words: Chickpea chlorotic dwarf virus, chickpea stunt complex, geminivirus, leafhopper transmission, non-propagative transmission, *Orosius albicinctus*, *Orosius orientalis*, persistent transmission

Introduction

Chickpea chlorotic dwarf geminivirus (CCDV) causes symptoms in chickpea plants which include stunting, phloem browning in the collar region, and, in the case of desi types, leaf reddening (Horn, Reddy, Roberts & Reddy, 1993). CCDV is widely distributed in several chickpea-growing areas in India and Pakistan (N M Horn, unpublished results). The symptoms are very similar, if not identical, to those associated with chickpea stunt disease, in India previously thought to be caused by bean leafroll luteovirus (BLRV) (Nene *et al.*, 1991). Elsewhere, similar symptoms in chickpea have been associated with other luteoviruses (Bosque-Perez & Buddenhagen, 1990; Duffus, 1979). Thus, it seemed likely that aphids, as vectors of luteoviruses, would be the only type of vectors involved in spreading chickpea stunt. The discovery that CCDV is also associated with chickpea stunt, and of its transmissibility by the leafhopper *Orosius orientalis* (Matsumura), is therefore new information on the ecology of the disease (Horn *et al.*, 1993).

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O. orientalis has been found on 12 plant species, including chickpea, in North India throughout the year (Bindra & Singh, 1970) and is considered to be economically important on sesame throughout India (Choudhary, Singh & Singh, 1986). The leafhopper has also been reported from Turkey (Lodos & Kalhadelen, 1985) and Egypt (Habib, El-Kady & Herakly, 1976; El-Nahal, Amar & El-Bohol, 1989). *O. orientalis*, earlier described as *Orosius albicinctus* (Ghauri, 1966), is also known to be the vector of five mycoplasma diseases in India, *viz.* aster phyllody (Rangaswamy, Suryanarayana, Muniyappa & Singh, 1988), groundnut witches' broom (Yang & Wu, 1990), potato purple top (Singh, Nagaich & Bhargawa, 1983), phyllody of *Sesamum* (Vasudeva & Sahambi, 1955), and sweet potato witches' broom (Yang & Chou, 1982). CCDV is the first virus reported to be transmitted by *O. orientalis*, in the Indian subcontinent (Horn *et al.*, 1993).

Information on the relationships between geminiviruses and their leafhopper vectors is very limited. Storey (1928) investigated the relationships between maize streak geminivirus (MSV) and its vector *Balclutha mbila* (= *Cicadulina mbila*), and Severin (1931), Freitag (1936) and Bennett & Wallace (1938) studied the transmission of beet curly top geminivirus (BCTV) by its vector *Eutettix tenellus* (= *Circulifer tenellus*). MSV and BCTV were shown to be transmitted in a persistent manner, and Freitag (1936) presented indirect evidence that BCTV does not multiply in its vector.

We have now examined the qualitative and quantitative characteristics of CCDV transmission by its leafhopper vector *O. orientalis* to better understand the epidemiology of the virus.

Materials and Methods

Virus isolate, insects and test plants

The virus isolate used was described by Horn *et al.* (1993). It was maintained in pea, *Pisum sativum* (cv. Bonneville). Leafhoppers were given an acquisition access period (AAP) of 3 days on infected pea plants (between 10 to 20 days after inoculation), followed by a 3-day inoculation access period (IAP) on healthy pea plants for virus propagation and maintenance. The culture of the leafhopper *O. orientalis* and its maintenance on *Crotalaria juncea* (sunnhemp), and *Sesamum indicum* (sesame) were also described by Horn *et al.* (1993).

The adult leafhoppers, used for the transmission studies, were from two different cultures. The original culture had a transmission rate (proportion of leafhoppers able to transmit) of 38%. The second culture used was the progeny of a single male and female from the original culture and had a transmission rate of 85%. For the determination of median and minimum transmission values, leafhoppers from the original culture were used. For all other experiments leafhoppers from the culture with the high transmission rate were used. The experiments were carried out in a glasshouse at temperatures between 25°C and 32°C. CCDV-infected pea plants (cv. Bonneville) were used for virus acquisition, and healthy pea plants for inoculation in all transmission tests. Inoculated test plants were scored for external symptoms and tested by ELISA, usually 14–20 days after the start of IAP.

Determination of minimum acquisition access period (AAP_{min}), minimum inoculation access period (IAP_{min}), and minimum latency period (LP_{min})

To determine the AAP_{min}, groups of 50 leafhoppers were given varying AAPs on three infected pea plants followed by an IAP of 4 days on three healthy pea plants. To assess the IAP_{min}, leafhoppers were given an AAP of 4 days and then starved for 2 h prior to

transferring them in groups of 50 leafhoppers to three healthy pea plants for varying IAPs. The LP is defined as the time between the start of the AAP and the end of the first IAP in which the insects were able to transmit the virus. To determine the LP_{min}, a group of 100 leafhoppers was given an AAP of 1 h, and transferred serially to pea seedlings at 1 h intervals.

Determination of AAP_{50}, IAP_{50} and LP_{50}

To determine AAP_{50}, leafhoppers were given an AAP of 1, 3, 8, 24 or 48 h. After each AAP, 50 leafhoppers were transferred individually to healthy pea seedlings for an IAP of 3 days. The leafhoppers were recovered and their sex determined. To determine IAP_{50}, leafhoppers were given a 4-day AAP. They were then starved for 1 h, and groups of 50 leafhoppers were given an IAP of 0.5, 1, 3, 8 or 24 h. They were confined individually to healthy pea seedlings for the duration indicated. The insects were recovered after the IAP and their sex was determined.

To determine the LP_{50}, leafhoppers were given a 14 h AAP, followed by five successive IAPs. During the IAPs, individual leafhoppers were kept on pea seedlings, a new seedling for each IAP. The first four IAPs were 8 h and the last one 42 h, to determine the maximum transmission.

For calculation of the median values of AAP, IAP and LP, the method described by Sylvester (1965) was used. The time was converted to $\log_{10}$ (time). The transmission percentages were transformed by putting the transmission percentage for the longest period tested at 100%. This permitted compensation for the exposed leafhoppers which were not able to transmit the virus. The converted percentages were transformed to probits, and then a linear regression of the probit value against $\log_{10}$ (time) was carried out.

Test for non-persistent transmission and transmission by nymphs through moulting

Two groups of 50 leafhoppers were given a short AAP immediately followed by a short IAP on three healthy pea plants per group, to check for possible non-persistent transmission. To test whether nymphs of *O. orientalis* can transmit the virus, and if it persists through moulting, 15 nymphs were given a 2-day AAP, and then transferred serially at daily intervals to healthy pea seedlings. The dates on which the nymphs moulted were recorded.

Serial transmission

Leafhoppers were given a 2-day AAP and then transferred individually to healthy pea seedlings at 1-day intervals, except on Sunday, to study their ability to serially transmit the virus to healthy pea plants until death.

Virus detection by ELISA

DAS-ELISA (Clark & Adams, 1977) was used for the detection of the virus in pea plants. Plates were coated with CCDV IgG (2 μg/ml for detection in plants, and 1 μg/ml for detection in insects) in carbonate buffer (pH 9.8). The antigen was extracted using phosphate-buffered saline (pH 7.2), containing 0.05% Tween 20 and 2% polyvinylpyrrolidone (= extraction buffer). For antigen extraction from insects, Nonidet P40 (NP40) was added to the buffer at 2 ml/litre. Extracts from single leafhoppers were prepared in 200 μl buffer, clarified at 8000 *g* for 10 min, and 100 μl of the supernatant was added to ELISA plates and incubated overnight at 4°C. The alkaline phosphatase conjugate was used at 2 μg/ml for detection in plants and at 0.5 μg/ml for detection in insects. For virus detection in plants, p-nitrophenyl phosphate (0.2 mg/ml) was used as a substrate, and absorbance readings were taken at 405 nm with a Titertek Multiskan. For detection in insects, a more sensitive

Table 1. *Number of plants infected with CCDV out of three pea plants inoculated with 50 leafhoppers after varying AAPs and IAPs*

		Number of plants infected			
AAP	IAP	Expt 1	Expt 2	Expt 3	Expt 4
2 min	4 days	3	3		
5 min	4 days	3	3		
10 min	4 days	3	3		
30 min	4 days	3	3		
24 h	4 days	3	3		
4 days	2 min			1	0
4 days	5 min			2	0
4 days	10 min			1	0
4 days	60 min			3	3
4 days	48 h			3	3

enzyme-amplification procedure was used (Van den Heuvel & Peters, 1989) and absorption values were recorded at 492 nm.

Quantitative virus assay in leafhoppers

One hundred leafhoppers were given a 3-day AAP on infected pea plants, and then transferred to groundnut, *Arachis hypogaea*, a non-host of CCDV (Horn *et al.*, 1993) on which the leafhoppers survive. Individual leafhoppers were tested by ELISA immediately after the 3-day AAP, and other individual leafhoppers were tested 9 days after transfer to groundnut. Leafhoppers, which were allowed to feed for 3 days on virus-free pea plants, were treated in a similar manner and served as controls.

Results

AAP_{min}, IAP_{min} and LP_{min}

The results of the transmission experiments are summarised in Tables 1 and 2. An AAP of 2 min, an IAP of 2 min, and an LP of 2 h still resulted in transmission of the virus.

Table 2. *Number of plants infected with CCDV out of three pea plants inoculated with 100 leafhoppers after a 1-h AAP and successive IAPs*

	Number of plants infected	
IAP	Expt 1	Expt 2
1–2 h[a]	0	2
2–3 h	0	2
3–4 h	2	2
4–5 h	2	3
5–6 h	1	2
6–7 h	0	2
7–24 h	3	3
24–72 h	3	3

[a] Time interval after start of AAP

Table 3. *Calculated AAP_{50}, IAP_{50} and LP_{50} (all expressed in hours) for the* O. orientalis – *CCDV relationship*

	Rep[a]. 1	Rep. 2	Rep. 3	Average (± SE)	
AAP_{50}	7.3	8.4	8.2	8.0	(0.4)
IAP_{50}	1.7	0.7	4.7	2.3	(1.2)
LP_{50}	26.3	31.4	25.4	27.7	(1.9)

[a] Replicate

Therefore the AAP_{min}, IAP_{min} and LP_{min} are likely to be shorter than 2 min, 2 min and 2 h, respectively. Even an AAP of 2 min resulted in good acquisition of the virus by its vector. For efficient inoculation, more time appears to be required, since the number of plants infected decreased when the IAP was shorter than 1 h (Table 1). The transmission efficiency decreases when the LP is less than 7 h (Table 2).

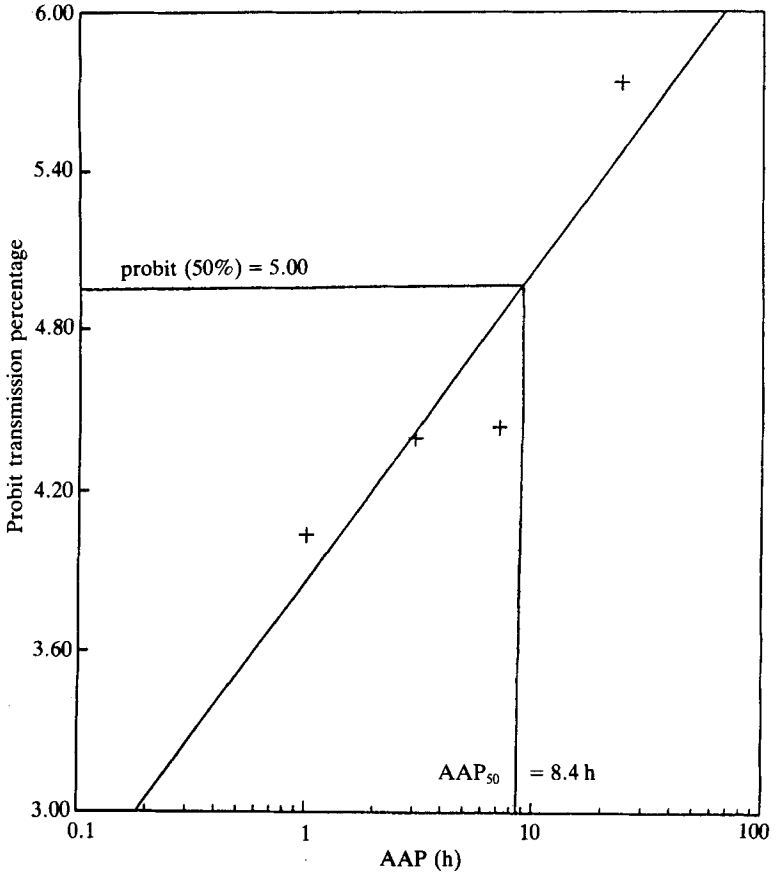

Fig. 1. Example of linear regression of the transmission percentages after probit transformation to determine the median acquistion access period (AAP_{50}) of CCDV transmission by *O. orientalis*. In this instance the estimated AAP_{50} was 8.4 h (replicate 2).

AAP_{50}, IAP_{50} and LP_{50}

The values determined in nine independent experiments are summarised in Table 3. As an example, the linear regression of the transmission percentages transformed to probits is given in Fig. 1, for one of the AAP_{50} experiments. The average values for AAP_{50}, IAP_{50} and LP_{50} were 8.0 h, 2.3 h and 27.7 h, respectively.

Test for non-persistent transmission and transmission by nymphs through moulting

None of the two groups of 50 leafhoppers transmitted the virus after a 10-min AAP followed by a 10-min IAP without a LP. Thirteen of the 15 nymphs tested, transmitted the virus as nymphs and did so for 2–3 days. Then they moulted and continued to transmit the virus as adults. Thus, they retained their transmission ability through moulting.

Transmission rates of male and female leafhoppers

The sex of 795 individual leafhoppers used in the experiments described above was determined: 39% were males and 61% were females. Of the 145 insects that transmitted the virus, 41% were males and 59% were females. Therefore males and females appear to have the same rate of virus transmission.

Serial transmission

The leafhoppers used in this experiment lived for 2–23 days after the AAP, although most survived 17–20 days. Most of the 60 leafhoppers tested, transmitted the virus until their death or till a few days before they died (e.g. LH54; Table 4). Some of them had a few interspersed failures in transmission (e.g. LH37, LH48, LH51; Table 4). A few leafhoppers stopped transmitting the virus long before they died (e.g. LH11; Table 4). Very few leafhoppers failed to transmit because a colony with a high transmission rate (85%) was used.

Quantitative virus assay in leafhoppers

The virus titre of individual insects, determined by ELISA, varied widely. The average absorbance values (± standard errors) of the leafhoppers fed on infected plants (exposed leafhoppers) was 0.252 (± 0.018) immediately after the 3-day AAP and 0.056 (± 0.040) 9 days later (12 days after start of the AAP). For the leafhoppers fed on virus-free plants

Table 4. *Range of variation in serial transmission of CCDV by seven leafhoppers selected from 60 tested after daily transfers to pea test plants*

	1[a]	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23
LH25[b]	−[c]	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−[d]							
LH11	+	+	+	−	−	−	−	−	−	−	−	−[d]											
LH18	+	+	+	+	+	+	+	+	+	+	+	−	−	−[d]									
LH37	+	+	+	+	+	+	+	+	+	+	−	+	+	−	−	+	+	+	−	−	−	−	−[d]
LH48	+	+	+	+	+	+	+	+	+	+	−	+	+	−	−	−	−[d]						
LH51	+	+	+	−	+	+	+	+	+	+	+	+	+	+	−	+	+[d]						
LH54	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+[d]			

[a] Serial number of daily transfer to pea test plants after initial 2-day AAP
[b] Code number of leafhopper
[c] + = pea plant infected
− = pea plant not infected
[d] Death of leafhopper

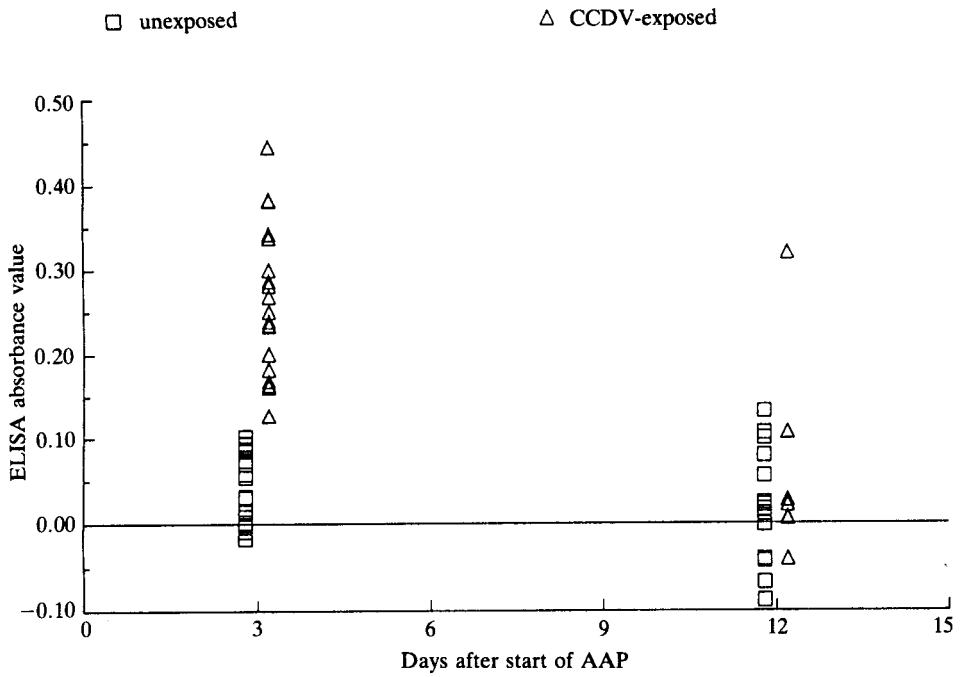

Fig. 2. ELISA absorbance values of unexposed and CCDV-exposed leafhoppers immediately after a 3-day AAP and after another 9 days on a non-host of CCDV. Every square or triangle represents the absorbance value for one leafhopper. Absorbance values measured at 492 nm, 30 min after addition of the final substrate; the average value of six buffer controls was subtracted.

(unexposed leafhoppers) these values were 0.047 (± 0.010) and 0.022 (± 0.018), for 3 and 12 days, respectively. These results are shown in Fig. 2. There were substantial differences in OD values of exposed and unexposed leafhoppers immediately after the 3-day AAP. However, 9 days later many of the exposed leafhoppers gave ELISA readings similar to those of the unexposed leafhoppers and only a few of the exposed leafhoppers still gave values that were substantially higher than comparable controls.

Discussion

The minimum values for AAP, IAP and LP found in this study represent extremes, whereas median values can be quantified better, and are ecologically more important, than minimum values. Although *O. orientalis* can acquire CCDV and inoculate the virus into a plant in short access periods, longer periods are needed for efficient transmission. Nevertheless, *O. orientalis* can be considered an efficient transmitter of CCDV, as especially shown in serial transmission tests by the persistence of the virus in the vector. In similar experiments with MSV there were many more interspersed failures during a period of transmission (Storey, 1928).

In studies on transmission of other geminiviruses, minimum values are generally given (Table 5). The minimum values now reported for CCDV agree with those given for beet curly top geminivirus (BCTV) (Severin, 1921, 1931; Bennett & Wallace, 1938). BCTV and

Table 5. *Minimum values of AAP, IAP and LP for the leafhopper transmission of geminiviruses as reported in this and other studies*

Virus[a]	Vector	AAP_{min}	IAP_{min}	LP_{min}	Reference
MSV	*C. mbila*	1 h	nd[b]	12 h	Storey, 1928
		15 sec	5 min	nd	Goodman. 1981
BCTV	*C. tenellus*	2 min	10 min	4 h	Severin, 1921, 1931
		1 min	1 min	5 h	Bennett & Wallace, 1938
BSDV	*O. argentatus*	nd	nd	48 h	Bowyer & Atherton. 1971
CCDV	*O. orientalis*	2 min	2 min	2 h	This publication

[a] MSV = maize streak virus, BCTM = beet curly top virus, BSDV = bean summer death virus, CCDV = chickpea chlorotic dwarf virus.
[b] not determined.

CCDV can both be acquired and inoculated very quickly. This would indicate that either the leafhoppers can reach the phloem rapidly, or CCDV and BCTV are not phloem limited.

Secondary spread within a crop can occur readily when the vector requires a relatively short time for acquisition and inoculation and the virus has a short latency period. Secondary spread has not been widely studied for leafhopper-transmitted pathogens (Chiykowski, 1971), but it is now known that both BCTV and CCDV can be acquired and inoculated in very short periods of feeding. This indicates the potential of CCDV to reach high infection levels in crops.

CCDV is retained by *O. orientalis* for up to 21 days (Table 4). Moreover, *O. orientalis* does not lose the virus through moulting. These results and the non-transmission of CCDV in a 10-min AAP immediately followed by a 10-min IAP suggest that CCDV is transmitted by *O. orientalis* persistently rather than non-persistently.

Loss of ability to transmit CCDV 10 or more days after acquisition (Table 4) provides indirect evidence that CCDV does not multiply in its vector. Furthermore, the reduced virus concentration on the 9th day after the AAP as compared to immediately after a 3-day AAP, suggests that the virus does not multiply in *Orosius orientalis* (Fig. 2). Indeed, the majority of leafhoppers from CCDV-infected plants gave absorbance values similar to those of leafhoppers from virus-free plants 9 days after the AAP. The serial-transmission experiment (Table 4) showed that the majority of leafhoppers still transmitted the virus 9 days after AAP. The amount of virus present in leafhoppers 9 days after the AAP must therefore have been below the detection level. Loss of virus during feeding on a non-host of the virus is compatible with non-propagative, merely circulative transmission. BCTV was shown indirectly to be transmitted non-propagatively by *E. tenellus* and the proportion of insects transmitting the virus gradually decreased when the vector was confined to a non-host of the virus (Freitag, 1936). Bennett & Wallace (1938), when indirectly assaying *E. tenellus* for BCTV, also found that the virus content in the insects decreased with increasing time on maize, when transferred to maize after feeding on infected beet. The non-propagative character of MSV transmission was shown in infectivity tests and ELISA by Reynaud & Peterschmitt (1992), who also reported that the concentration of the virus decreased in most of the insects, although a few did retain high virus concentrations.

This is the first report of median values for a leafhopper-transmitted virus. The median values found for CCDV transmission are in the same range as those of the persistent transmission of potato leafroll virus (PLRV) by *Myzus persicae* (AAP_{50} 12 h, IAP_{50} 45–105 min, LP_{50} 24–124 h; Peters, 1986; Van den Heuvel, Boerma & Peters, 1991). The median IAP values for transmission of both PLRV and CCDV varied widely.

Female *O. orientalis* are not more efficient transmitters of CCDV than males in contrast to results of early studies on MSV and its vector *Cicadulina mbila* (Storey, 1928, 1932). Our results with CCDV are in agreement with those of Freitag (1936) for BCTV, who found that males and females of *E. tenellus* were equally efficient transmitters.

The above facts lead to the conclusion that CCDV is transmitted by the leafhopper *O. orientalis* in a persistent, non-propagative and circulative manner. This leafhopper is an efficient vector of CCDV; it can transmit the virus even after short feeding periods and the virus persists even up to 21 days after acquisition. The transmission characteristics of CCDV by its vector resemble more closely those of BCTV, another member of the same sub-group of geminiviruses, than those of MSV, a member of another sub-group of geminiviruses.

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