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Evaluation of Low Temperature Induced Mutants of *Cucumber green mottle mosaic virus* for Cross-protection in Cucurbits

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Abstract

Cucumber green mottle mosaic virus has been reported as the most prevalent and dominant virus infecting cucurbit crops in KPK, Pakistan. Inoculated plants of *C. sativus* were treated at low temperature to generate and select mild strain of CGMMV for cross protection. Among 200 inoculated plants during mild strain selection, only two were showing no symptoms and were also strongly positive in serological assay as well as in electron microscopy. These two isolates, designated as Pk-47 and Pk-81, were selected for subsequent experiments on cross-protection.

In greenhouse experiments, the control plants started developing symptoms just 5 days after inoculation and were exhibiting severe symptoms in 10-12 days post challenge inoculation. Plants inoculated with Pk-47 isolate initially developed very mild symptoms that disappeared in one week post inoculation period. In cross-protection test, with the two selected isolates, symptoms developed in plants that were challenge-inoculated 5 or 7 days post protection-inoculation. Those plants that were given challenge-inoculation 10 days after the protection-inoculation did develop some mild symptoms, but later on they showed recovery. In all other treatments (challenge-inoculation done after 15, 20 and 25 days post protection-inoculation) both the Pk-47 and Pk-81 mild isolates were exhibiting convincing protection abilities to control the severe strain of CGMMV-Pk. The effectiveness of the cross-protection was evaluated at three different temperatures (17-22°C, 22-27°C and 27-32°C), and it was observed that it worked equally well under all the experimental conditions. Among the two isolates, protection abilities of Pk-81 seemed to be more promising comparing to Pk-47. All the plants remained symptom-less, for almost two months, after the challenge inoculation. The experimental results revealed that protection inoculation at least 15 days prior to challenge inoculation will serve better.

Keywords: Cucurbit; CGMMV; Incidence; Tobamovirus; Pakistan

Introduction

It has long been known that the activity of one plant virus can prevent or delay the infection of closely related viruses [1]. The phenomenon that was coined cross-protection, pre-munity, acquired immunity, antagonism, cross immunization or interference, needs the former virus to be established for conferring the effective resistance. It is a type of induced resistance, developed in plants, by the systemic infecting virus against very closely related strain. Ideally, the protecting strain is not aggressive, but inhibits the pathogenetic effect of the super-infecting strain introduced by challenge inoculation [2,3]. It was described as early as 1930s, and was independently confirmed by several virologists. Cross-protection has also been demonstrated for other classes of plant pathogens and at times it was believed to be analogous to the immunity developed in mammals following vaccination, although its mechanism does not involve the formation of antibodies [4].

Palukaitis and Zaitlin [5] proposed that the positive sense RNA of protecting viruses would sequester the minus-strand of super-infecting virus strain, provided they have homology high enough to allow the annealing of the two strands. This annealing of negative strand also further blocks the replication of incoming virus. It was only this model that could explain the protection offered by naked RNA or viroids, and also, it justify why high level of homology is required to induce this response. Several lines of evidences indicate that RNA-based protection is derived from a nucleotide (nt) sequence-specific host defense mechanism, termed as post-transcriptional gene silencing (PTGS) that targets viral RNAs for destruction [6,7] demonstrating that PTGS is a common plant defense response active against diverse viruses and likely to play an important role in cross-protection. Data presented by different scientists also revealed that pre-expression of viral proteins play an important role in cross-protection. The relative importance of the different mechanisms may be a function of the viruses and the particular host species. Rezende et al., [8] suggested that the host may play a role in cross-protection between tobamoviruses.

Cross-protection has effectively been used against *Papaya ringspot virus* (PRSV) [9-11], *Watermelon mosaic virus* (WMV) [12], *Cucumber mosaic virus* (CMV) [13-15], *Tobacco mosaic virus* (TMV) [16-18], *Citrus tristeza virus* (CTV) [19], *Zucchini yellow mosaic virus* (ZYMV) [20], *Cowpea mosaic virus* (CPMV) [21] and *Tomato mosaic virus* (T_oMV) [22].

Etiology of the viral disease of cucurbit crops, prevalent in the cucurbit growing regions of Pakistan, clearly indicates the dominance of *Cucumber green mottle mosaic virus* (CGMMV). This high incidence could be attributed to the contact transmission of the virus, coupled with poor phytosanitary measures prevalent in the area. This necessitates testing of possible control strategies to eliminate the disease from the cucurbit growing areas. We are reporting here the cross-protection offered by mild isolate of CGMMV-Pk, induced by low temperature treatment, against the severe strain. Our efforts resulted in selection of two isolates, which were multiplying at high rate

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and were almost symptom-less in the inoculated plants. They offered effective protection, when challenge inoculated by the severe parent virus. As CGMMV is a contact transmitted virus, hence it will be easy to inoculate the seedlings with very little efforts and expenditures and hence will prove to any effective tool in devising an effective integrated control program for this menace.

Materials and Methods

Virus isolate

The CGMMV-Pk was isolated from *Lagenaria siceraria* samples collected at Khyber Pakhtunkhwa Province, Pakistan in 2002 and was maintained on mechanically inoculated plants of *Cucumus sativus* in insect proof greenhouse at Utsunomiya University, Japan. Mechanical inoculations were performed by rub inoculating the healthy *C. sativus* plants as described previously (Ali et al.). The plants were kept in greenhouse at 22-27°C with supplemented fluorescent lights.

Local lesion host assay

Chenopodium amaranticolor, C. quinoa, Nicotiana benthamiana, N. Glutinosa, N. Rustica, C. sativus and *Vigna unguiculata* were mechanically inoculated as described previously (Ali et al.) in an attempt to find out any reasonable local lesion hosts for mild strain selection, after the low temperature treatment.

Mild strain selection at low temperature

The inoculated plants of *C. sativus* were kept in greenhouse at a temperature of (12-16°C) for 45 days. Meanwhile, healthy seeds of *C. sativus* were germinated in 200 plastic pots, each having two seeds, to raise the seedlings for mild strain selection. After germination thinning was done to reduce the number of seedling to one each pot. Mechanical inoculation was done by grinding 1 g leaf in 50 ml of 0.05 M potassium phosphate buffer, pH 7.5 and test plants were rub inoculated on carborundum (600 mesh) dusted leaves. The plants were kept in greenhouse at 22-27°C with supplemented fluorescent lights. This temperature was previously evaluated as best for replication and symptom expression in *C. sativus* plants. The plants were grown for more than 6 weeks after inoculation. Indexing was started routinely after 10 dpi and any plant showing symptoms was discarded.

Serological assay

All the remaining plants were tested for the presence of CGMMV using the dot immuno-binding assay (DIBA) protocol [23]. The reaction was recorded visually by the intensity of the colour. Only those plants having high concentration of virus particles and no visible symptoms were selected for cross-protection test.

Electron microscopy

Electron microscopy was also done for the selected samples to confirm the results of serological assay. Sap from infected leaves was coated on carbon-formvar coated grids as described previously [24] and were examined with JEOL 100S electron microscope.

Cross-protection test

C. sativus seedlings were mechanically inoculated with the selected isolates (hereafter termed Pk-47 and Pk-81) by rub inoculation as described previously by Ali et al., [24]. Infection was confirmed by DIBA assay 10 days after inoculation. Severe parent isolate that was used for mild isolate induction was challenge inoculated. Infectivity of the extract was determined by inoculation of healthy *C. sativus* seedlings. Challenge inoculation was applied on upper leaf 5, 7, 10, 15, 20 and 25 days after the inoculation of mild isolate. Three different temperatures (17-22°C, 22-27°C and 27-32°C) were tried for cross-protection assay and at each temperature three plants were used. Breakdown of the protection was judged by the appearance of typical severe mosaic symptoms on test plants, which were kept in the greenhouse for at least two months, after challenge inoculation.

Results

Selection of mild strains

Cucumber green mottle mosaic virus has been reported as the most prevalent and dominant virus infecting cucurbit crops in KPK, Pakistan [24] inducing mosaic symptoms that later on turns into yellow mosaic (Figure 1a), mottle and severe puckering. In an attempt to obtain mild isolate for cross-protection, assay hosts including *C. amaranticolor, C. quinoa, N. benthamiana, N. Glutinosa, N. Rustica, C. sativus* and *V. unguiculata* were mechanically inoculated with CGMMV. No satisfactory local lesion host could be found in this assay. Hence *C. sativus* was finally selected to be used as host for low temperature treatment and also for the mild strain selection. After low temperature treatment, the isolate was rub inoculated onto 200 healthy *C. sativus* seedlings at single leaf stage and were kept in greenhouse under flruorescent light. Regular selection was done on the basis of symptom development. Almost half of the plants, being grown for mild strain selection, were discarded due to appearance of severe symptoms in the normal course of time. Out of the remaining plants (97), 15 were having no symptoms and were negative in serological assay (DIBA), indicating that these plant actually, for some reason, escaped infection with the mild strain. Also, no virus particle could be detected in samples from these plants in electron microscopy. Thirteen plants were

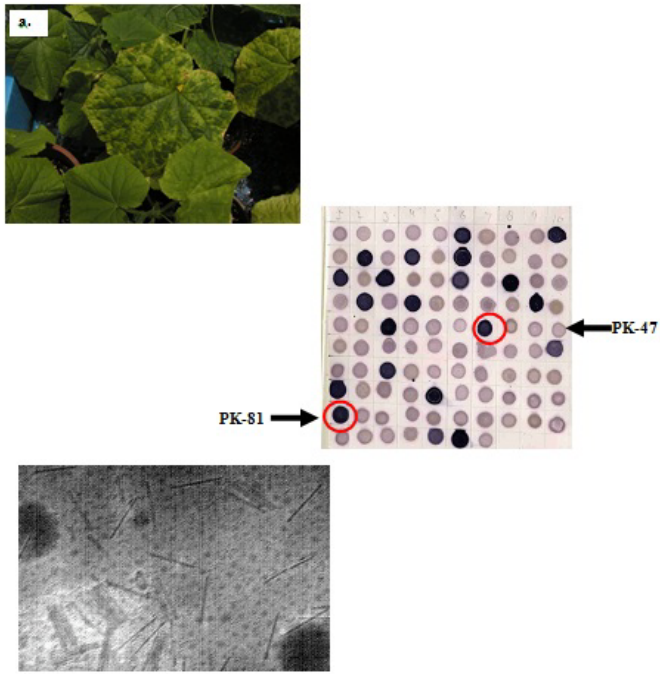

Figure 1: (a) Symptoms produced by Cucumber green mottle mosaic virus on *C. sativus* plants. (b) Serological assay (DIBA) showing the results of mild strain selection of CGMMV-Pk isolate treated at low temperature (12-16°C). Isolate number 47 and 81 were giving very strong positive signal in DIBA assay, but no symptoms on *C. sativus* plants. (c) Electron micrograph of *C. sativus* sample infected with mild strain Pk-47, showing large number of CGMMV viral particles.

showing moderate symptoms of green mosaic and were positive in DIBA assay also. Around 48 plants were showing very mild symptoms, but also very mild positive dot in the serological assay, indicating that the mild symptoms are just because of low virus concentration. Very strong positive results in DIBA analysis were observed from 21 plants. Among these 21 plants, only two were showing no symptoms and very strong positive result in serological analysis (Figure 1b). When checked under electron microscope, both the plant samples were having very high concentration of virus particles (Figure 1c). These two isolates, designated hereafter as Pk-47 (Figure 2a) and Pk-81(Figure 2b), were selected for subsequent experiments on cross-protection.

Cross-protection under greenhouse conditions

In greenhouse experiments, the control plants started developing symptoms just 5 days after inoculation and were exhibiting severe symptoms in 10-12 days post challenge inoculation (Figure 3c). Plants inoculated with Pk-47 isolate initially developed very mild symptoms that disappeared in one week post inoculation period. In cross-protection test, with the two selected isolates, symptoms developed in plants that were challenge-inoculated 5 or 7 days post protection-inoculation. Those plants that were given challenge-inoculation 10 days after the protection-inoculation did develop some mild symptoms, but later on they showed recovery and the symptoms disappeared from the 3rd or 4th leaf after challenge-inoculated leaf. In all other treatments (challenge-inoculation done after 15, 20 and 25 days post protection-inoculation) both the Pk-47 and Pk-81 mild isolates were exhibiting convincing protection abilities to control the severe strain of CGMMV-Pk. The effectiveness of the cross-protection was evaluated at three different temperatures (17-22°C, 22-27°C and 27-32°C), and it was observed that it worked equally well under all the experimental conditions (Figure 3a and 3b). Among the two isolates, protection abilities of Pk-81 seemed to be more promising comparing to Pk-47. All the plants remained symptom-less, for almost two months, after the challenge inoculation. Observations could not be prolonged as limited growing media and rapid growth of the host plants hindered continuation. After two months natural senescence of the plants could be seen in negative control as well as in the protected plants. The experimental results revealed that protection inoculation at least 15 days prior to challenge inoculation will serve better. Due to time limitation, experiments could not be planed to test the efficiency of protection under different disease pressures as well as to figure out the beneficial effects of control on yield and yield components, nor could it be evaluated under natural field conditions. This pilot study provided a good nucleus material that can be used in subsequent experiments to fully characterize the isolates, before recommending it for commercial growth.

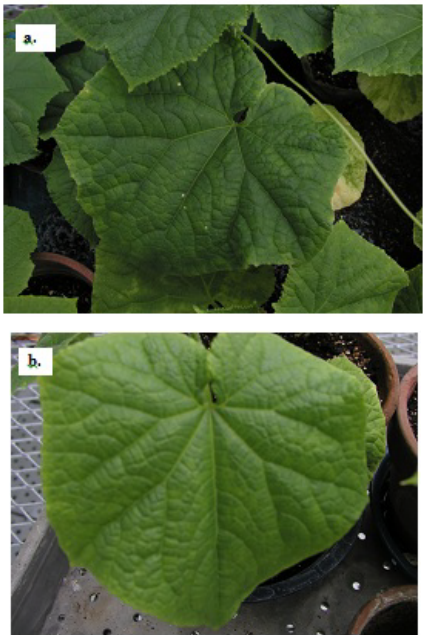

Figure 2: Mild strains of CGMMV-Pk selected at low temperature (12-16°C) treatment for 45 days. (a) Isolate Pk-47 and (b) Isolate Pk-81.

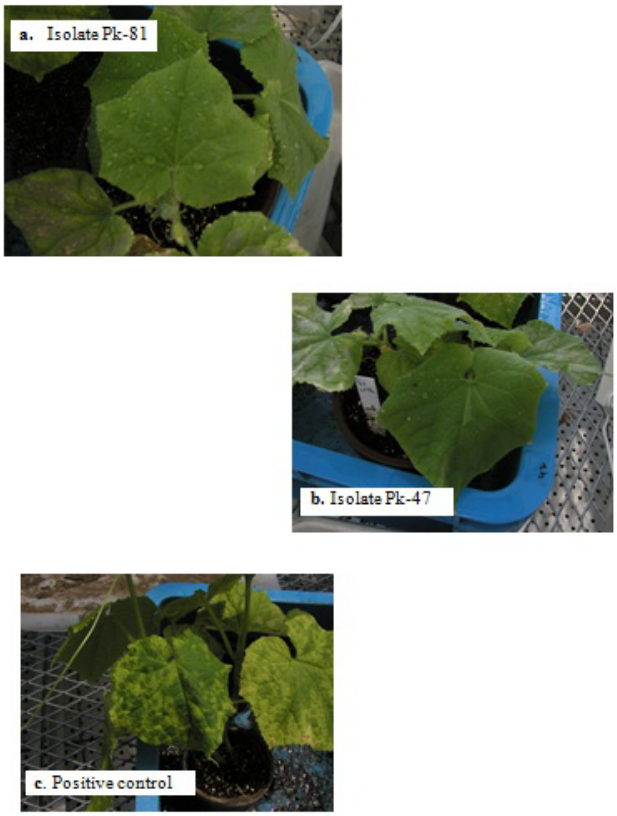

Figure 3: Efficacy of cross-protection abilities of two mild isolates, (a) Pk-47 and (b) Pk-81, induced by low temperature treatment in *C. sativus* plants. (c) Severe strain of CGMMV inducing characteristic symptoms on *C. sativus* in positive control.

Discussion

Etiology of the viral disease of cucurbit crops, prevalent in KPK, Pakistan, revealed that the mosaic disease is caused by complex of different viruses. Five viruses were found infecting the crops, with CGMMV being the most widespread [24]. The dominance of CGMMV could be attributed to the contact transmission of the virus, coupled with the poor phytosanitary measures. The farmers in the area have little or no knowledge of viral diseases or their mode of transmission and spread. Most of the farmers use their own seeds or seeds from uncertified agencies without due regard for the health and purity of the seeds. The connecting irrigation channels among the fields and use of contaminated tools exacerbate the situation.

Cross-protection has long been demonstrated as an effective control for plant viruses as well as other classes of plant pathogens and at times it was believed to be analogous to the immunity developed in mammals

following vaccination, although its mechanism does not involve the formation of antibodies [4]. PTGS is a common plant defense response active against diverse viruses and likely to play an important role in cross-protection [6,7]. Low temperature treatment has been found effective to induce mutation [13]. We successfully obtained two mild isolates Pk-47 and Pk-81 from the severe strain of CGMMV-Pk by subjecting it to low temperature treatment. It has been observed that a certain time interval between the protection and challenge-inoculation is required to establish cross-protection, and the degree of cross-protection is related to the concentration of the protecting virus in the plants [10,11,25]. Our preliminary data also supports this conclusion however; it needs to check the effect of inoculum's concentration (both protecting and challenge isolate) in our case.

One of the major concerns in using mild strain for cross-protection is the effect that this virus may cause in association with other viruses under natural field conditions and also on other host crops in the vicinity. Therefore, there is a dire need to evaluate this type of synergistic association with other viruses, before recommending it for control in commercial farmer's fields. Multiple inoculations of mild isolates of different viruses have been investigated and were found effective [14]. Efforts could be directed to induce mild strains for other prevalent viruses also, to help farmer controlling the complex of viruses under field conditions or at least minimizing the detrimental effects to economic beneficial level.

Another limitation for making cross-protection as the method of choice is the excessive labour cost and long time required to inoculate mild strain for protection purposes. CGMMV is a contact transmitted virus, once the mild strains are fully characterized, they can easily be used on large scale for protection with least cost. Even seed treatment may provide an easy and feasible mean of inoculating the crops. Spray gun can also be used for effective inoculation of the plants at seedling stage and this method would avoid the possibility of mechanical transmission of any severe strains of the virus present in the seedlings population. Also, in this case if few seedlings escape spray inoculation with the mild strain, they would be subsequently infected during the cultural practices from the adjacent abundant source of inocula. The stability of the mild strain in this case should not be a concern as tobamoviruses are known to have the most stable genome among the plant viruses, but one must always need to take the necessary precautions by continually evaluating the actual inocula used for cross-protection. Although, the previous findings Ali et al., [24] indicate very low level of diversity in the sequence of CGMMV isolates reported so far, one must also investigate the diversity of the virus in the area concerned. Some sort of variability do occur in genome of CGMMV, which is also reflected by different symptom expressions reported for watermelon isolate from Japan and CGMMV-Pk. Watermelon strain recorded in Japan [26] induce local lesions in *C. amaranticolor,* but CGMMV-Pk did not produce any such symptom in *C. amaranticolor*. Low diversity mean effective control, on the other hand, if there is a diverse population in the area it would probably make cross-protection a bit ineffective. It has been demonstrated that mild PRV-HA 5-1, which provided the CP gene of the line 55-1 from Hawaii, do not offer protection against PRV isolates from Thailand and only limited protection against those from Taiwan [9,27].

Genetic resistance and use of transgenic material would be the priority method to control, if available, but use of cross protection is also effective and excellent control have been reported in many viral diseases; PRSV [9,11,27], WMV [12], CMV [14,15], TMV [16-18], CTV [19], ZYMV [20] and T_oMV [22]. Moreover, the reluctance of consumers in using the harvest of genetically modified crops will make it a preferred strategy for controlling viral diseases. Careful attention to selection of the best protective isolates of virus and their introduction into the crop to be protected are essential. We would like to characterize these two isolates under natural field conditions to study their effect on the adjacent crops, and also their interaction with the prevalent viruses in the region. Once established, these mild isolates can subsequently be used at commercial scale for controlling the prevalent CGMMV infection in cucurbit crops of the region. Other control strategies may also be tried along with the mild strain protection to formulate an effective integrated approach for successfully controlling the mosaic disease in the cucurbit crops of Pakistan.

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