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Citrus Tristeza Virus-*p23* Gene Correlated with the Pathogenicity in Non Citrus Hosts

Hala A. Amin* and B.W. Falk*

* *Virus and Phytoplasma Res. Dept., Plant Pathol. Res. Inst., ARC, Giza, Egypt.*

** *Plant Pathol. Dept., Univ. of California, Davis, USA.*

In this study, to gain a deeper insight into the role of *p23* on CTV pathogenesis, we tested the influence of *CTV-p23* gene on *Nicotiana benthamiana*, a plant that does not support CTV infections (CTV nonhost). The *CTV-p23* gene was inserted in several configurations into a heterologous Potato virus X (PVX) vector. Systemic infection with replicative mRNA for each of the recombinant PVX-p23 constructs elicited severe necrotic symptoms with necrotic lesions on inoculated *N. benthamiana, Datura. stramonium and tomato,* leaves after 6 dpi and leads to death of the plant after 8-12 *dpi*. In the meantime the PVX-p23 antisense showed mosaic symptoms as with the PVX-wild type vector. In addition, the *p23* gene elicited the formation of local necrotic lesion in *Nicotiana glutinosa, Nicotiana tabacum* cv. Turkish and *N. tabacum* cv. Havana. In an effort to understand the mechanism of the induced pathogensis and the contribution of *CTV-p23* gene in development of necrotic symptoms in *N. benthamiana*., we examined whether the necrosis symptom elicited as a result of *CTV-p23* RNA transcribed accumulation and /or the *CTV-p23* protein expression. Northern blot assays revealed that all PVX constructs showing intensive bands corresponding to high level accumulation of the plus genomic and sg PVX *m*RNA as a consequence of virus replication increasing. Relative quantitative real time PCR results revealed that the accumulation of the viral RNAs [genomic and subgenomic *m*RNA (sgRNA)] does not related with the necrotic symptoms regarding to the PVX-WT as a normaliser. The statistical data showed that there are no significant variants between all the PVX constructs, and all of them are completely different than the healthy control data. Our results showed that the defective mutants (truncated and frameshift *p23*) indicate that the exogenously expressed *p23* proteins are functionally active. Site directed mutagenesis to disrupt and restore potential base pairing in the zinc finger domain sequence suggest that the zinc finger domain is indispensable for *p23* function in *vivo* and for the necrotic symptoms development (pathogensis) and the necrosis could be due to expression of a *p23* protein or a truncated protein that has a conserved zinc finger domain (active site) of the *p23* protein.

Keywords: CTV, gRNA, northern blot, *p23*, PCR, PVX vector, site directed mutagenesis and sgRNA.

Citrus tristeza Virus (CTV) is distributed world wide and is the causal agent of one of the most economically important diseases of citrus. CTV, a member of the genus *Closterovirus* within the family Closteroviridae, is phloem limited and is

transmitted by aphids in a semi-persistent manner. CTV virions are flexuous filaments of 2000 X11 nm in size. The CTV genome is a single- stranded positive sense RNA of 19,226 to 19,296 nucleotides (nts) that contains 12 open reading frames (ORFs) and 5' – and 3' – terminal untranslated regions (UTRs) (Pappu *et al.*, 1997). The only CTV protein with no homologue in other closteroviruses is *p23* which is an RNA-binding protein (L´opez *et al.*, 2000). In citrus protoplasts its sgRNA is most abundant at the beginning of the infection and the second most prevalent in later stages, suggesting a role of *p23* in early steps of viral replication or transcription (Hilf *et al.*, 1995; Navas-Castillo *et al.*, 1997). However, *p23* accumulates at low levels in citrus –infected plants (Pappu *et al.*, 1997). In vitro, *p23* has ability to bind RNA in a non – sequence – specific manner, and mutations affecting the cystein and histidine residues of a zinc finger domain conserved in different isolates increase the dissociation constant of the *p23* – RNA complex (L´opez *et al*, 2000). Additionally, *p23* is involved in controlling asymmetrical accumulation of positive and negative strands during RNA replication, with zinc finger domain and an adjacent basic region being indispensable for asymmetrical accumulation of the plus strand (Satyanarayana *et al.*, 2000) Furthermore, transgenic Mexican lime plants constitutatively expressing *p23* of the severe CTV strain T36 display alterations resembling the symptoms induced by CTV in this host, with their intensity being associated with *p23* accumulation (Ghorbel *et al.*, 2001). This strongly suggests that this protein is an important pathogenicity factor. Recently, proteins *p25*, *p20*, and *p23* have been found to act as RNA silencing suppressors in *Nicotiana benthamiana* and *Nicotiana tabacum* plants (Lu *et al.*, 2004) and *p23* has been involved in symptom production in *Citrus* and related species (Fagoaga *et al*. 2005 and Ghorbel *et al.*, 2001).

The PVX vector has already proved efficient for the study of different aspects of plant/pathogen interactions (Rommens *et al.*, 1995; Hammond-Kosack *et al.*, 1995; Scholthof *et al* 1995; Culver, 1996). Although this system has been especially designed to transiently express foreign genes, it can also be used to study the properties of a foreign noncoding RNA sequence (Culver, 1996).

Real-time RT-PCR techniques have been widely used in studies on gene expression and/or regulation and detection of specific nucleic acids present in different types of samples, as well as in specific virus diagnosis and quantification (Bustin, 2000 & 2002; Freeman *et al.*, 1999; Heid *et al.*, 1996; Mackay *et al.*, 2004; Tan *et al.*, 2004). The high sensitivity, specificity, and reproducibility provided by real-time RT-PCR without the need for post-PCR sample processing, are the main reasons justifying their application for pathogen detection and comparison.

In this study, we analyzed the influence of *CTV-p23* gene on disease symptoms in *Nicotiana benthamiana*, a plant that does not support CTV infections (CTV nonhost) and systemically infected and local lesion hosts.

Materials and Methods

This work has been done in Dr. Falk, B.W. Lab. Pathology Dept., University of California, Davis, 95616. USA.

PVX constructs cloning and in vitro transcription and plant inoculation:

In an effort to understand the mechanism of the induced pathogensis and why *CTV-p23* gene developed systemic necrotic symptoms in *N. benthamiana* and whether the necrosis elicited as a result of *CTV-p23* RNA transcription or the *CTV-p23* protein expression, The *CTV-P23* gene was isolated and amplified from El-Kanater (K1) isolate (Egyptian isolate) as described previously (Sambade *et al.*, 2003; Amin *et al.*, 2004; Roy *et al.*, 2006). The *CTV-p23* gene was inserted in several configurations into PVX- vector (a modified viral vector) as shown in Fig. (1). The *CTV-p23* gene without ATG start codon (truncated gene) was cloned

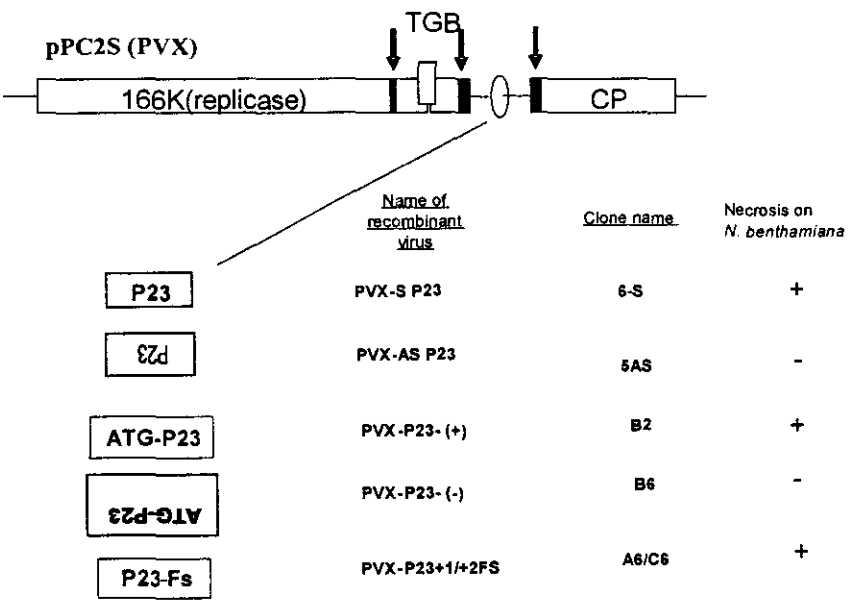

Fig. 1. Schematic representation of the PVX vector with different *CTV-p23* configurations.

into PVX-vector (pPC2S) to create PVX-p23 with two different orientation, sense (PVX-Sp23 (+) and antisens (PVX-ASp23 (-) which referred as 6-S and 5AS respectively. In addition, the *CTV-p23* coding region with ATG start codon was cloned into PVX vector (pPC2S), in two opposite orientation (sense (PVX-p23-S(B2) and antisense (PVX-p23-AS(B6) (Fig. 1). To achieve that, the *p23* gene was amplified using two newly designed primers, PVX-Eco-F and PVX-p23 R (Table 1) specific for *CTV-p23* coding region with suitable end addition restriction enzymes (*EcoR*V) at the 5' and 3' ends, respectively. We also constructed the PVX vector with *p23* with +1 frame shift sequence (PVX-p23+1FS referred as (A6 and C6) (Fig. 1) using the same newly designed primer with *EcoR*V end addition and with only one nucleotide addition between the start codon and the 1st nucleotide after the

start codon in the sense primer (the Bold nucleotide) using FPVX-F and PVX-p23 R (Table1). Clones were confirmed by nucleotide sequence analysis. The PVX-p23 constructs were subjected to T7 *in vitro* transcription reaction using mMessage mMachine kit (Ambion) .for synthesis capped replicative PVX -infectious RNAs. The Infectious RNAs were generated from 1-2ug of *spe1* linearized recombinant vector. Inoculation was done by mixing 20 µl of transcript with 100 µl of FES buffer (1% sodium pyrophosphate, 1% bentonite, 1% celite in 0.1M glycine, 0.06M dibasic potassium phosphate) and three *N. benthamiana* plants were inoculated for each construct. The inoculated leaves were harvested at *6* days post inoculation *(dpi)* and confirmed for the presence of the desired insert sequence by RT-PCR using PVX1 and PVX2 primers (Table1). Leaves were frozen at -80 C and used as a source of inoculum for all the experiments. The inocula were prepared for inoculation of 10 *N. benthamiana* plants (4-6 leaf stage) by the previously collected leaf material in 0.03M potassium phosphate buffer. pH 7.0, containing 0.1 % sodium sulfite.

Table1. Oligonucleotides used in PCR for making and detection of transgene constructs

Primers	len	Seq 5`-3`
PVX-ECO-F	33	ATCGGATATCATGGATGATACTAGCGGACAAAC
FPVX_R	31	ATCGGTCGACTCAGATGAAGTGGTGTTCACG
FPVX_F	34	ATCGGATATCATGAGATGATACTAGCGGACAAAC
PVX-p23-F	33	ATCGGTCGACATGGATGATACTAGCGGACAAC
PVX-p23-R	31	ATCGGATATC**TCA**GATGAAGTGGTGTTCACG
18SF	15	TACGCCCCGCCCAAA
18SR	21	CACTGGCAGTCCTTCGTGAGT
25K-PVX-Forward	24	CATACAGAAGCCAGGACCTATTCC
25K-PVX-Reverse	22	TCCTTGTGGTGTTGTCCAAAGT
MuHA-F	40	ATAGGAGTGTACGTGGATAGAGGTAGAAAACTCG ACAAGG
MuHA-R	40	GCGCCTTGTCGAGTTTTCTACCTCTATCCACGTAC ACTCC
Pvx1	23	CCATTGCCGATCTCAAGCCACTC
Pvx2	24	GTAGTTGAGGTAGTTGACCCTATG

The symptoms were monitored daily and all the plants were screened for the presence of the recombinant PVX virus using regular RT-PCR after 6 *dpi*. Five different hosts (*Datura. stramonium* and tomato, *Nicotiana. glutinosa, Nicotiana tabacum* cv. Turkish and *N. tabacum* cv. Havana) other than *N. bentamiana,* were used for inoculation with all new PVX constructs to demonstrate its effect and the symptoms development. 6 replicate from each host for each PVX construct (treatment) were inoculated with PVX inocula collected from *N. bentamiana* previously inoculated with the *in vitro*-transcribed replicative RNA for all PVX-constructs.

RNA analysis:

To determine the effect of *CTV- p23* gene on PVX expression and to check if the necrotic symptoms are due to protein expression or RNA accumulation, Northern blot assays briefly were performed using RNAs from systemic infected leaves showing symptoms collected at 7 *dpi*. Total RNA was extracted from leaves showing systemic symptoms by using an RNeasy Plant Mini kit (Qiagen, USA.). Approximately 5 µg RNA was subjected to electrophoresis in 1% agarose containing HEPES-EDTA. Following electrophoresis for 1.5 hr at 100V, RNA was blotted onto Hybond-NX membrane (Amersham Biosciences). The *p23* gene was ligated in pBluescript SK+ vector by using the two restriction enzymes *sal*1 and *EcoR*V. A linearized pBluescript vector containing the *CTV-p23* gene (1 µg) was labelled with ^{32}P using T7 and T3 Maxitranscript, Ambion kit for synthesis of + and - RNA probe. RNAs were normalized based on the amount of rRNA on the gel (Kevil *et al.*, 1997).

Protein expression analysis using relative quantitative real time PCR (RRt-PCR):

Total RNA was extracted from *N. bethamiana* leaves showing systemic symptoms by using an RNeasy Plant Mini kit (Qiagen, USA.). Trace DNA contamination was removed from total RNA preparations by DNase treatment using 2 U DNase I (Fermentas) in a 20 µl reaction mix for 25 min at 37 °C. The reaction was stopped by addition of 1 µl of 25 mM EDTA and incubation for 20 min at 65 °C. The DNase treated total RNAs were subjected directly to cDNA synthesis using 5 µl of treated RNA in total volume of reverse transcription (RT) RT- reaction mix for the cDNA synthesis. the RT- mix was conducted using 1.25 µl of 0.5 µg random hexamers primer (invitrogen, USA.) and 0.1 µl of each of the superscript II (invitrogen) and RNase out followed by incubation at 42°C for 1hr, 94°C for 3 min. .The cDNA products were diluted until 200µl total volume. 5 µl of the diluted cDNA was subjected directly to the relative quantative real time PCR reaction using Aplied Biosystem SYBR green 2X mix. The reaction was conducted in 25 µl total volume using 10 pmol of the PVX-25K gene specific primers (25K-PVX-forward and 25K-PVX-Reverse, Table 1) and 18SrRNA specific primers (18SF&18SR-Table1) as a plant endogenous control for relative quantification. The real time PCR reaction was performed under recommended reaction conditions. RRt-PCR quantification according to Applied Biosystem instruction (50°C for 2 min, 95°C for 10 min and then 40 cycles of 95°C for 15 sec and 60°C for 1 min . RRt-PCR was based on relative abundance, as determined by relative threshold cycle (Ct) value compared with plant18SrRNA endogenous control Ct. Three biological replicates were used for each constructs beside two technical replicates for each plant and appropriate controls containing no template were included in our experiments. The linearized Ct was calculated according to the following formula according to Bustin (2000 and 2002):

Relative Ct (rel Ct) = Ct(target) – Ct (ref endogenous cont.)
Normalized Ct (Norm Ct)= rel(Ct) – Cal (Ct)
Calibrator= relCt for the PVX –WT.
Linearized Ct (LCt)= 2^-((Norm Ct)

Site directed mutagenesis of the PVX-p23 sense coding construct:

The PVX-p23 clones with mutation was carried out using two new designed *p23* mutated primers containing the mutation sites, MuHA-F and MuHA-R, Table (1) (according to zinc finger domain nucleotides and amino acid alignment of *p23* gene with different configuration Fig. 10) at nucleotide No. 205, 207 and 229. The PCR reaction was carried out using Turbo PFU polymerase at 95C for 1 min and 18 cycles at 95°C for 30 sec, 60°C for 1 min and 68°C for 20 min. with final extension at 68°C for 7 min. The PCR product was undergoing ligation and transformation according to Sambrook *et al.* (1989). The clones were screened for the presence of the mutated *p23* gene using PCR and DNA sequence analysis.

Results and Discussion

The *p23* gene of *Citrus tristeza virus* has been implicated as a suppressor of virus induced gene silencing in *N. benthamiana* and *N. tabacum* plants (Lu *et al.*, 2004) and as an RNA-binding protein (L'opez *et al.*, 2000) Additionally, *p23* is involved in controlling asymmetrical accumulation of positive and negative strands during RNA replication, with zinc finger domain and an adjacent basic region being indispensable for asymmetrical accumulation of the plus strand (Satyanarayana *et al.*, 2000). Furthermore, the *p23* protein is an important pathogenicity factor in *Citrus* and related species but not on nonhosts *N. tabacum* and *N. benthamiana* (Fagoaga *et al.*, 2005 and Ghorbel *et al.*, 2001).

In this study, we tested the effects of *CTV-p23* gene on *N. benthamiana*, a plant that does not support CTV infections (CTV non host). In an effort to understand the effect of the *CTV-p23* gene on *Nicotiana benthamiana*, the PVX-p23 constructs (Fig. 1) were subjected to T7 *in vitro* transcription reaction using mMessage mMachine kit (Ambion, USA.) for synthesis capped replicative PVX -infectious RNAs. Systemic infection of each of the recombinant PVX viruses revealed that the expression of *CTV-p23* from the PVX vector (6-S) elicited necrotic lesions on the inoculated leaves plus a severe systemic necrosis on newly formed *N. benthamiana* leaves after 6 *dpi*. In the meantime the (5AS) showed mild mosaic as the *N. benthamiana* inoculated with the PVX vector (PVX- WT) as shown in Fig. (2).

All PVX- p23 chimeric constructs were subjected to DNA sequence analysis. Again, all the chimeric PVX-p23 construct were subjected to T7 *in vitro* transcription reaction. 10 plants of *N. bentamiana* were inoculated with the chimeric PVX replicative RNA and the symptoms phenotypes were monitored after 6 *dpi*. The PVX- *p23* constructs either with sense or +1 frameshift elicited a severe necrotic symptoms with necrotic lesions on inoculated leaves after 6 *dpi* and leads to death of the plant after 8-12 *dpi*. The PVX -p23 with antisense orientation PVX-p23-AS (B6) showed only mild mosaic on *N. benthamiana* newly formed leaves as the PVX-WT control, (Fig. 2). Theses results were unexpected, as recombinant PVX vectors usually induce milder symptoms than non recombinant PVX (Chapman *et al.*, 1992 and Hammond-Kosack *et al.*, 1995).

Fig. 2. *N. benthamiana* inoculated with PVX-p23 constructs showing Small brown necrotic lesions appeared on both inoculated and newly formed leaves at 6 *dpi*. A: inoculated plant with PVX-p23. (sense 6-S) . B: PVX-p23 (antisense-5AS) inoculated plants. B2:: inoculated plant with PVX-p23 sense orientation. A6 and C6):: *N. benthamiana* inoculated with PVX-p23 construct with +1 frameshift mutation) . B6: inoculated plants with PVX-p23 antisense orientation showing no necrotic symptoms. PVX-WT: PVX wild type.

Initial screening for the presence of the PVX –p23 construct inside the plant after inoculation was conducted using convenient regular RT-PCR with two newly designed primers specific for the PVX vector upstream and downstream the cloning site, PVX1 and PVX2 (Table 1) these primers were without end addition enzyme restriction sites to facilitate the detection using the RT-PCR analysis for the phenotypic results. The detection was proceeded directly after six days post PVX construct inoculation to avoid the PVX recombination. All the examined plants inoculated with PVX-p23 constructs gave an amplified products at 850 bp as expected, whereas the PVX-WT control inoculated plants gave only 200 bp amplified product as shown in Fig. (3). This result indicated that no significant insert deletion had occurred in the PVX-p23 constructs progeny while such deletions are a common occurrence when propagating recombinant PVX due to PVX recombination (Chapman *et al.*, 1992 and Hammond-Kosack *et al.*, 1995).

Five different hosts (*D. stramonium* and tomato, *N. glutinosa, N. tabacum* cv. Havana and *N. tabacum* cv. turkish) other than *N. bentamiana,* were used for inoculation with all new PVX constructs to demonstrate its effect and the symptoms development; Table (2) summarized the symptoms for all PVX constructs observed on different hosts.

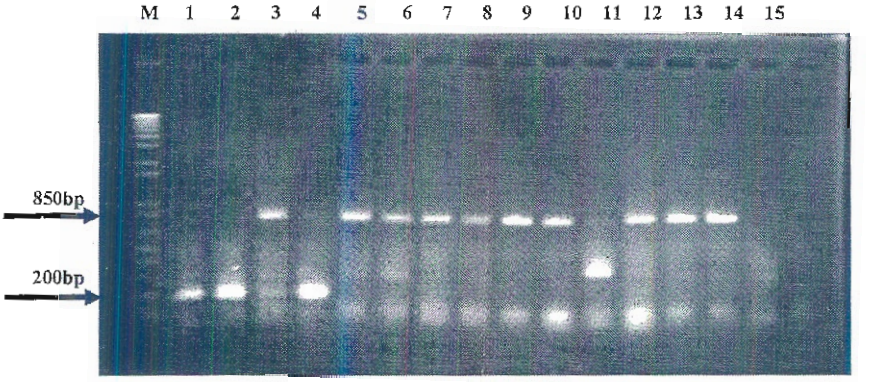

Fig. 3. RT-PCR from total RNA extraction of inoculated *N. Benthamiana* with different PVX constructs. Lane M: 1 Kb ladder (invitrogen), Lane 1&2: PVX-WT, Lane 3&4: RT-PCR product from PVX-p23 sense with ATG (B2), Lane 5,6,7 &8 : are amplified product from PVX_p23 with frame shift (A6 and C6 consequently), Lane 9, 10, 11 & 12: PVX-p23 antises with full coding gene (B6 and non coding gene (5AS). Lane 13 & 14: PVX-p23 sense orientation without ATG (6-S), Lane 15: RT-PCR for the *N. benthamiana* uninoculated plant.

Table 2. Symptoms produced as a result of PVX construct introduction on different hosts

Host	Transcript inoculation and symptom expression						
	PVX-p23 (6-S)	PVX-p23 (5AS)	PVX-*p23*-AS (B6)	PVX-p23+1FS (A6 & C6)	PVX-p23 S (B2)	PVX- WT vector	No PVX const
N. bentamiana	N. on inocul. & uninoc.	M.	M. on uninoc.	NL. on inocul. & N. on uninoc.	NL. on inocul. and N. on uninoc. & D. 12*dpi*	M.	H.
N. tabacum cv. Turkish	NL.	H.	H.	NL. on inocul.	NL. on inocul.	Chl. on inocul. & M. on uninoc.	H.
Datura	N.	H.	Chl. on inocul. & M. on uninoc.	NL. on inocul. & N., M. on uninoc. with C6	NL. on inocul. & M. on uninoc.	M. & VC NL. but tiny & few on *inocul.*	H.
N. tabacum cv. Havana	NL.	H.	H.	NL.	NL.	M.	H.
N. glutinosa	N.	H.	H.	NL. on inocul.	NL. on *inocul.*	M.	H.
Tomato	N. & BL.	H.	M.	N. & BL. & LC then D	N. & BL.	M.	H.

N: necrosis Inocul.: inoculated leaves D: death Uninoc: uninoculated leaves
NL: necrotic lesion VC: vein clearing M: mosaic H.: no symptoms
Chl: chlorotic spots LC: leaf curling BL: blackening

To determine the effect of *CTV- p23* gene on PVX expression and if the necrotic symptoms due to *p23*-RNA transcribed accumulation and / or the *p23* protein expression., Northern blot assays were performed using RNAs from infected plants showing systemic symptoms collected at 7 *dpi*. Fig. (4) represent the schematic PVX genomic organization and the expected subgenomic RNAs size with the inserted gene. All PVX - constructs were showing intensive bands corresponding to the plus genomic and subgenomic mRNA after 2hr X- ray film exposure using the T3- *p23* cRNA specific radioactive P^{32} prob. Whereas they were showing faint bands corresponding to the minus g & sg RNA using the T7 hRNA probe specific for the *p23* gene (Fig. 5 A, B &C) this means that the plus PVX recombinant virus RNA accumulated at high level. However, Northern blot could not reveal more knowledge about the *p23* gene expression, but the increase in PVX genomic and sgRNA accumulation was assumed to be a consequence of increase of virus replication. These results were in contrast with the Chapman *et al.* (1992) observation, who stated that the insertion of a foreign sequence in PVX usually has a debilitating effect on virus accumulation.

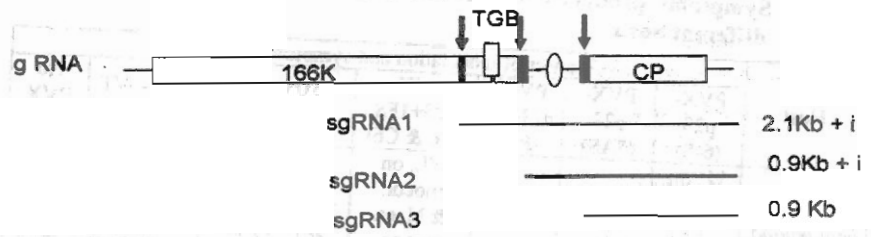

Fig. 4. Schematic representation for the PVX genomic RNA organization and the expected subgenomic RNAs size. i: means the size of the inserted gene

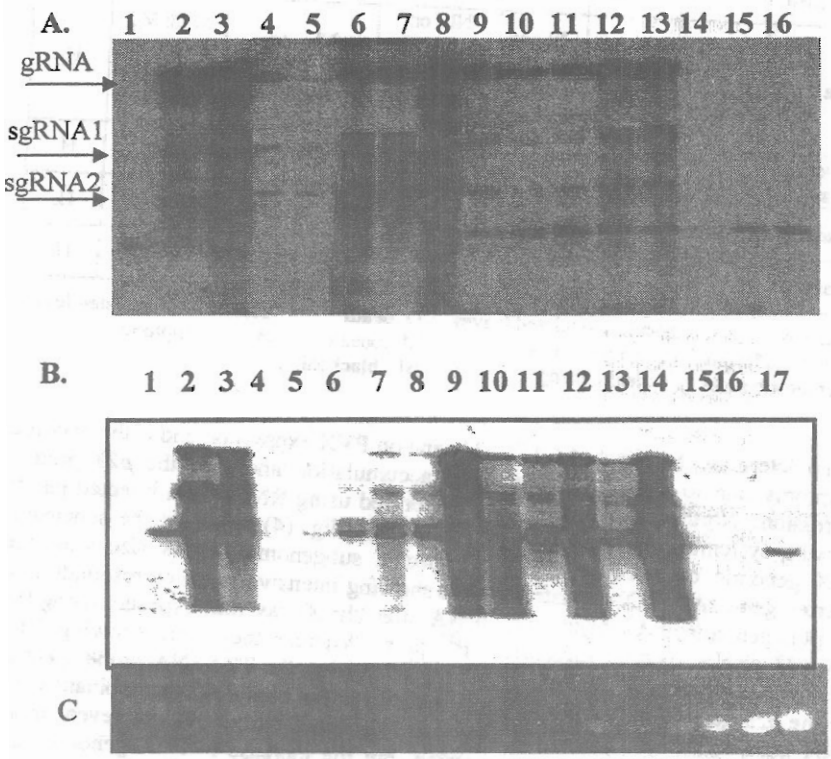

Fig. 5. Northern blot for total RNA extracted from *N. benthamiana* inoculated with different PVX constructs. A.: is Northern blot hybridized with ^{32}P labelled T7 *in vitro-* transcribed *p23* h RNA. B: Northern blot hybridized with ^{32}P labelled T3 *in vitro-* transcribed *p23* c RNA. Lane 1: RNA of Healthy *N. benthamiana* plant, Lane 2&3: PVX-p23-sens (no ATG), Lane 4&5: PVX-p23 (AS-non coding gene, Lane 6,7&8: PVX_p23 (AS-coding gene), Lane 9, 10,11 &12 : PVX-p23 (frameshift), Lane 13&14: PVX_*p23* –sense (with ATG-coding gene), Lane 15&16: PVX-WT, 17: TBSV- control C: The photographs of the ethidium bromide-stained RNA gel before transfer.

Gene expression levels are commonly determined using northern blot analysis. Real time PCR (Rt-PCR) is more rapid and sensitive and can be more specific than northern blot analysis. Determining the amount of template by PCR can be performed in two ways: as relative quantitation and as absolute quantitation. Relative quantitation describes changes in the amount of a target sequence compared with its level in a related matrix. Quantification can be difficult because many sources of variation exist, including template concentration and amplification efficiency (Bustin, 2002 and Freeman *et al.*, 1999). Several internal controls have been used in relative Rt-PCR. However, Morrison (1998) recognized that 18S rRNA sequences are highly conserved; therefore, the internal control was amplifying plant 18S rRNA from the recombinant PVX inoculated plants for underestimation of plant gene expression. SYBR green is a fluorogenic minor groove binding dye that exhibits little fluorescence when in solution but emits a strong fluorescent signal upon binding to double-stranded DNA. The important parameter for quantitation is the threshold cycle (Ct). The higher the initial amount of genomic DNA, the sooner accumulated product is detected in the PCR process, and the lower the C_T value.

In the present study, two primers PVX-RT-F and PVX-RT-R (Table 1) were used to detect the PVX g-RNA and sg-RNA1 and 2 as indicated in schematic representation (Fig.4) amplifying plant 18S rRNA as internal control from the recombinant PVX inoculated plants for underestimation of plant gene expression. According to the linearized Ct values (LCt), The B2 (PVX-P23-Sense with ATG, *i.e.* the full coding gene) treatment was high (1.831594), followed by B6 (PVX-p23 antisense coding gene) with a LCt value (0.805636) and then the 6-S (PVX-p23-sense without ATG with LCt= 0.721439).The frameshift construct (A6 and C6) showed lower linearized Ct value. These results showing that the accumulation of the viral RNAs (genomic and sgRNA) does not related with the necrotic symptoms. Because the frameshift constructs (PVX_p23-Fs (A6 &C6) showing severe reaction of necrosis on *N. bentamiana* although the level of the accumulation of the PVX RNA regarding to the PVX-WT as an normaliser (Calibrator), according to the linearized Ct data , was so low, in the meantime, the PVX-p23 –antisese (B6 showing higher linearized Ct value than the PVX-p23-sense (6-S) without start codon (Fig. 6), the statistical data showed that there is no significant variants between all the PVX constructs, and all of them is completely different than the healthy control data (data not shown).

All the chimeric *p23* nucleotide sequence were aligned and the deduced amino acid as Fig . (7) and it was found that there is one start codon for methionin amino acid before directly the zinc finger domain. So we nocked the *p23* gene at this position using site directed mutagenesis via long range PCR and using mutated primers, Fig. (8) show the amplified product for mutated PVX-p23 clone in sense orientation. The infected plants with mutated PVX-p23 constructs did not show any necrotic symptoms (no significant symptoms) data not shown.

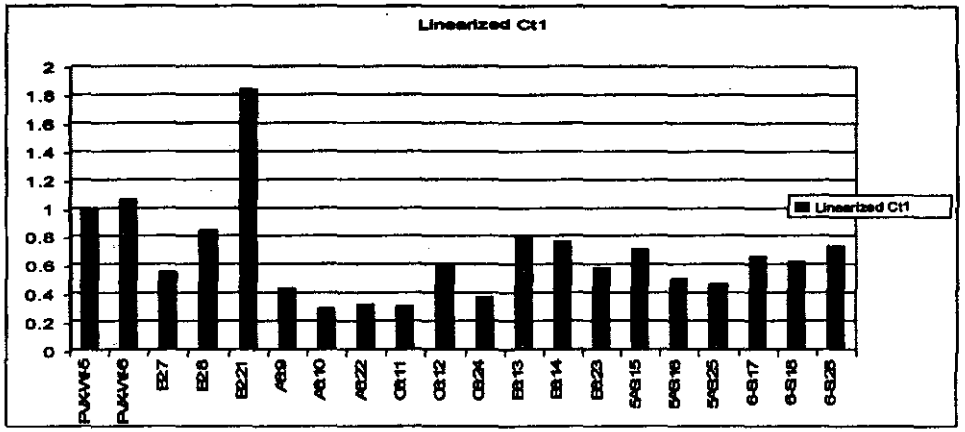

Fig. 6. Chart showing the Linearized Ct values for all the PVX- constructs in comparison to the PVX-WT as a calibrator. B2: represents PVX-p23-S (with ATG), A6 &C6: PVX-p23+1FS (A6 and C6) +1 frame shift, B6 & 5AS: PVX-ASp23 (-).

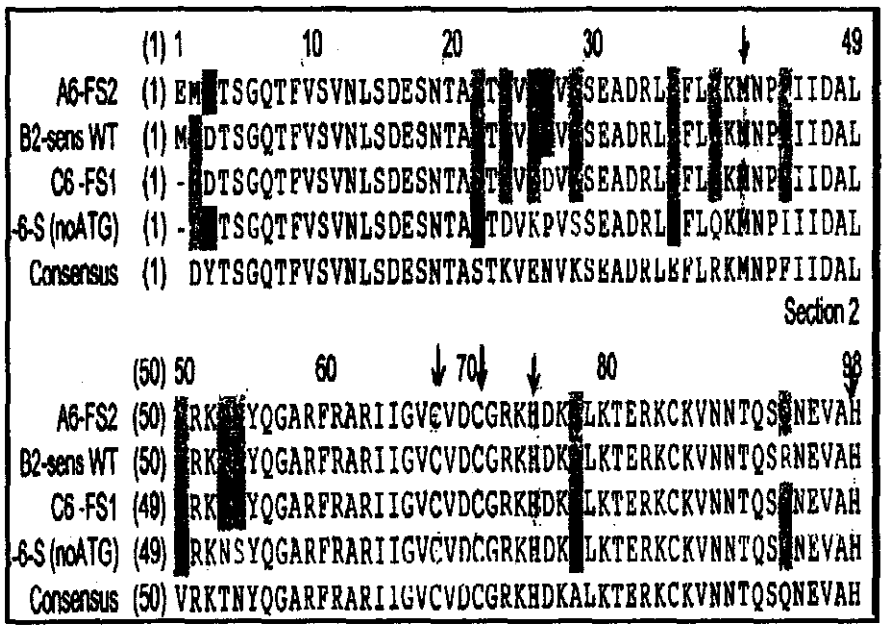

Fig . 7. A: Protein alignment of *p23* gene with different configuration. The arrow show the site of the zinc finger domain amino acids .

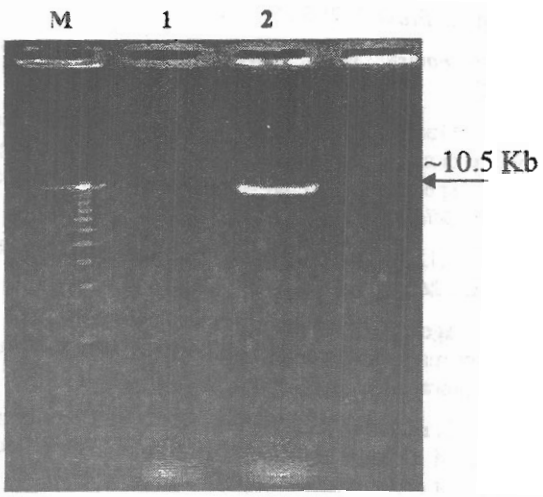

Fig. 8. The amplified product for mutated PVX-p23 clone in sense orientation. M: 1 kb lader, Lane 1: water control, Lane 2: the amplified product for the full length PVX-p23 sense (B2) clones using the mutated primers.

Therefore, from these data, It could be concluded that the zinc finger domain is indispensable for *p23* function *in vivo* and for the necrotic symptoms development (pathogensis) and the necrosis could be due to expression of a *p23* protein or a truncated protein that has a conserved zinc finger domain (active site) of the *p23* protein.

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جين ال *p23* لفيروس التدهور السريع للموالح له علاقه بالمرضيه فى عوائل اخرى خلاف الحمضيات

هاله عبدالله أمين* و بريس فولك**

* قسم بحوث الفيروس- معهد بحوث أمراض النباتات- مركز البحوث الزراعية.

** قسم أمراض النباتات- جامعة كاليفورنيا- ديفيز- الولايات المتحده الأمريكيه.

للتعرف بصوره أكبرعلى دور جين ال*p23* لفيروس التدهور السريع فى الموالح (*CTV*) و أرتباطه بامرضيه تمت دراسة تأثير هذا الجين عند حقنه فى نباتات الدخان من النوع *Nicotiana benthamiana* ، كنبات غير عائل لفيروس التدهور السريع . تم أدخال الجين *p23* لفيروس *CTV* لخمسة تحورات مختلفة فى ناقل فيروس البطاطس X (heterologous PVX-vector). استحثت العدوى الجهازيه بال RNA المتضاعف لكل تراكيب ال PVX المعاد تركيبها مع جين ال *p23* ، ظهرت أعراض حاده فى صورة نقط محليه ميته على نباتات ال *N. benthamiana* ، *Datura stramonium* و الطماطم المحقونه بعد ٦ أيام من الحقن، كما أنه أدى الى موت النبات بعد ٨-١٢ يوم من الحقن. فى ذات الوقت، فأن ال PVX-p23 الأتجاه المعاكس أظهر أعراض موزيك على نباتات *N. benthamiana* مطابقه تماما للأعراض الناتجه عن PVX-WT السلاله البريه من الناقل PVX ، بالإضافه الى أن الجين *p23* أستحث تكوين بقع موضعيه ميته على أوراق نباتات *Nicotiana glutinosa* ، *Nicotiana tabacum* cv. Turkish ، *N. tabacum* cv. Havana.

و فى محاوله لفهم ميكانكية استحداث المرضيه و دور جين *CTV-p23* فى تطور أعراض الموت على نباتات *N. benthamiana* . تم أختبار إذا كان عرض الموت (necrosis) ناتج عن تراكم *CTV-p23* RNA أوعن التعبير البروتينى لجين *CTV-p23* ، فأن أختبار Northern blot نتج عنه أن كل تراكيب PVX أظهرت حزم كثيفه تعبر عن تراكم شديد للحمض النووى genomic RNA الموجب و subgenomic mRNA (sgRNA) كدليل إيجابى على حدوث تضاعف الفيروس. استخدام تقنية Relative Quantitative Real time PCR أظهرت النتائج أن تراكم RNA للفيروس (genomic and sgRNA) ليس له علاقه بمرض الموت بالمقارنه بال PVX-WT الذى أعتبر كمقارنه (normaliser) و قد أظهرت نتائج التحليل الأحصائى أنه ليس هناك أختلافات معنويه بين تراكيب PVX المختلفه و كلهم مختلفين تماما عن نتائج النبات السليم (المقارنه). كما أظهرت النتائج أن الطفرات المعيبه (truncated and frameshift *p23*) ذات تأثير وظيفى فعال. كما أن الطفرات الموجهه لأعطاب و إستعادة القدره على إزدواج القواعد أظهرت أن منطقة zinc finger domain مسئوله عن و ظيفه الجين *p23* داخل النبات هى المرضيه و تطور أعراض موت الخلايا و أن هذا الموت قد يعزى إلى البروتين الناتج عن التعبير الجينى لجين *p23* أو truncated protein و الذى يحتوى على تتابع محافظ لمنطقه ال zinc finger domain لبروتين ال *p23* .