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AN ANALYTICAL STUDY ON UTILIZATION OF DS RNA FOR CONTROLLING STREAK MOSAIC VIRAL DISEASE IN SUGARCANE

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ABSTRACT

Sugarcane streak mosaic virus (SCSMV; Poacevirus; Potyviridae) causes streak mosaic disease in sugarcane (Saccharum inter specific hybrids), a major industrial crop widely cultivated for sugar and ethanol production in tropical and subtropical regions. All equipment used should be sanitized to prevent the spread of the sugarcane mosaic virus. When it comes to viral disease, fungicides and other pesticides have been shown to be ineffective. Plant host resistance is the most effective way to combat viral disease. The aim of the study is to study the utilization of dSRNA for controlling streak mosaic viral disease in sugarcane. As a result, the current study on SCSMV disease control in sugarcane will provide a foundation and significantly broaden the scope of virus disease control using an RNAi approach. The results showed that using bacterially produced dsRNA to silence the SCSMV CP gene is an effective way to control SCSMV in sugarcane. Total RNA was purified from sugarcane leaves as described. The procedure described was used to make first and second strand cDNA. This research also provides a reliable and potentially useful tool for studying gene silencing mechanisms in plant virus infections, as well as plant protection against virus diseases.

Keywords: *Sugarcane, Mosaic, Control, RNA, Disease, dSRNA, SCSMV etc.*

1. INTRODUCTION

Sugarcane mosaic virus (SCMV) is the most common virus that causes mosaic disease in sugarcane around the world. Many high-yielding cultivars are no longer grown becauseto their sensitivity to SCMV, which poses serious issues in many sugarcane-producing countries. When the incidence of infection was greater than 50%, Handojo et al. (1978) found that SCMV might impair sugar output by 10– 22%.

SCMV (Sugarcane Mosaic Virus) is a plant pathogenic virus belonging to the Potyviridae family. The virus was initially discovered in Puerto Rico in 1916, and by the early 1920s, it

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had spread throughout the southern United States. SCMV is a major source of worry due to its significant economic impact on sugarcane and maize.

Many factors influence the success and accuracy of diagnostic methods, but sample quality is often the most important, especially in large-scale routine indexing. When sampling plant sections or tissues examine the virus distribution throughout the plant as younger leaves typically have the highest virus content. After mechanical inoculation of leaves with SCMV, this research reports the detection of virus by RT-PCR in susceptible and moderately resistant sugarcane cultivars.

1.1 Mosaic disease

The mosaic virus is a parasitic infection that kills plants, gardens, and crops at the molecular level. If left untreated, a plant infected with the mosaic virus can spread the virus to other plants, potentially affecting an entire harvest. Roses, beans, tobacco, tomatoes, potatoes, cucumbers, pumpkins, squash, melons, and peppers are among the horticultural and vegetable crops affected by mosaic viruses.

Mosaic is a plant disease caused by hundreds of different virus strains. Mosaic infections can affect a variety of economically significant crops, including tobacco, cassava, beet, cucumber, and alfalfa. Tulip mosaic virus "breaks" tulip and lily blossoms, resulting in gorgeous and colourful streaks; this rare, distinctive impact fueled some of the 17th century's Tulip Mania craze.

Mosaic symptoms vary, but uneven leaf mottling is a typical sign (light and dark green or yellow patches or streaks). Veins may be lighter than normal or bordered with dark green or yellow, and leaves are commonly stunted, curled, or puckered. Plants are frequently dwarfed, having less fruit and blooms than typical, as well as being malformed and stunted. Mosaic symptoms are sometimes misdiagnosed as nutrient insufficiency or herbicide harm, especially at temperatures exceeding 27 °C (81 °F). Aphids and other insects, mites, fungus, nematodes, and touch propagate the causative viruses; pollen and seeds can also transport the infection. Mosaic can be avoided by utilizing virus-free seeds and plants, producing resistant cultivars, separating new and old plantings, rotating annuals, and adhering to strict sanitation and pest-control practices.

1.2 Sugarcane Mosaic Virus (SCMV)

Sugarcane mosaic infection (SCMV), having aplace with class Potyvirus, family Potyviridae, is a genuine microbe of numerous monocotyledonous harvests including sugarcane. In Pakistan, roughly 10–32% misfortunes have been assessed in sugarcane yield which brings about 6–10% decrease in sugar creation. Contaminated plants indicated mosaic example and are described by average pinwheel formed incorporation bodies in cell cytoplasm. The 10 kb single abandoned RNA genome of SCMV encodes a solitary polypeptide which is severed either co-or post translationally into ten develops proteins (P1, HC-Pro, P3, 6K1, CI, 6K2, VPg, NIa-Pro,
NIb, and CP). C-end of the polyprotein encodes the coat protein (CP) which encapsidates the viral genome through helical plan of its various subunits. Hc-Pro is a multifunctional protein and a proteinase, which is liable for the increase of infection genome, foundational infection development, and concealment of plant RNA hushing hardware by interfacing with various host factors

SCMV (Sugarcane Mosaic Virus) is a plant pathogenic virus belonging to the Potyviridae family. The virus was initially discovered in Puerto Rico in 1916, and by the early 1920s, it had spread throughout the southern United States. SCMV is a major source of worry due to its significant economic impact on sugarcane and maize.

1.3 Disease of the Sugarcane Mosaic

Sugarcane (Saccharum spp. hybrids), the world's most important sugar and energy crop, is a perennial, high biomass herb ratoon C4 crop that originated in the tropics. It is grownin over 100 nations or regions throughout the tropics and subtropics, covering around 27 million hectares. The global annual output of fresh cane is around 1.95 billion tonnes,

providing roughly 80% of sugar, 60% of bioethanol, and a total economic worth of $75 billion dollars. The pressed cane juice can also be used to make diesel, jet fuel, and other high-value products. Sugarcane by-products can be utilized for direct-fired power generation, field fertilizers, and fruit treeseedling culture substrate.

One of the most common viral sugarcane diseases is mosaic. After invading sugarcane, the virus causes systemic illness. Theincubation time is typically 10 days, but it can be as long as 20–30 days. The disease might appear as early as the second year after infection.

2. LITERATURE REVIEW

Bushra Tabassum (2022)for inducing virus resistance in plants, RNA-mediated silencing technology has emerged as the preferred method. The presence of double-stranded RNA (dsRNA), which is not only the product of RNA silencing but also one of the most potent triggers of RNA interference, is a key aspect of this technique (RNAi). These dsRNAs are sliced into short RNA pieces known as small interfering RNAs (siRNAs), which are hallmarks of RNAi, when RNAi is induced. The phenomena of RNAi can be used to provide significant virus resistance in transgenic plants. CEMB's creation of potato virus Y (PVY) resistant potatoes and sugarcane mosaic virus (SCMV) resistant sugarcane was used as an example in the current chapter. We're at the start of a new erain functional genomics, thanks to RNAitechniques.

Christine Rodriguez Guerrero (2021)the nucleic acid RNA (ribonucleic acid) is found in all living cells. RNA interference (RNAi) isa term that refers to a phenomenon in which RNA fragments bind to messenger RNAs that

code for proteins and prevent them from being translated. Saurabh, along with co- authors Vidyrthi and Prasad, stated in an article titled "RNA interference: concept to reality in crop improvement," published in 2014, that "the discovery of this phenomenon (RNA interference) has transformed into a powerful tool of genetic engineering and functional genomics." As a result, the RNAi phenomenon has the ability to unravel a wide range of biological issues and obstacles at the molecular level.

Chen, Jian-Sheng & Shan (2020) RNA silencing is one of the preserved antiviral instruments in plants, and viruses encode RNA silencing suppressors (RSS) to defeat have RNA silencing and encourage virus contamination. Sugarcane streak mosaic virus (SCSMV; species Sugarcane streak mosaic virus, variety Poacevirus, family Potyviridae) is a significant causal operator of sugarcane mosaic disease in numerous nations in Asia, including China. In this study, we utilized Agrobacterium co-invasion to show that the SCSMV P1 protein, instead of the assistant part proteinase (HC-Pro), capacities as a solid RSS to stifle nearby RNA silencing in Nicotiana benthamiana.

Fatima Yousif Gaffar and Aline Koch (2019) Viruses are committing parasites which cause a scope of extreme plant diseases that influence ranch efficiency around the globe, bringing about enormous yearly misfortunes of yield. Consequently, control of viral microorganisms keeps on being an agronomic and logical test requiring creative and historic procedures to satisfy the needs of a developing total populace. Throughout the most recent decade, RNA silencing has been utilized tocreate plants with an improved resistance to biotic burdens dependent on their capacity to give assurance from intrusion by unfamiliarnucleic acids, for example, viruses. This

characteristic wonder can be abused to control agronomically pertinent plant diseases.

Rosa, Cristina & Kuo et. al, (2018) The source of RNA interference (RNAi), the phonesentinel framework generally shared among eukaryotes that perceives RNAs and explicitly debases or forestalls their interpretation in cells, is proposed to originate before the last eukaryote basic predecessor (138). Of specific pertinence to plant pathology is that in plants, yet additionally in certain organisms, bugs, and lower eukaryotes, RNAi is an essentialand powerful antiviral safeguard, and late investigations have uncovered that little RNAs (sRNAs) associated with RNAi assume significant functions in other plant diseases,including those brought about by cell plant microbes.

3. MATERIAL AND METHOD

3.1 Treatment of sugarcane setts infectedwith SCSMV using extracted dsRNA

Sugarcane setts with single internodes weretaken from a SCSMV-infected sugarcane plant. 15 of the 30 sets were treated with extracted dsRNA, while the other 15 were left untreated as a control. The following protocol was used to treat infected setts with dsRNA.

- 2 litres of distilled water were combined with crude bacterial extractscontaining approximately 100 mg ofdsRNA.
- The sugarcane setts were immersed in the solution for three hours.
- After 3 hours, these setts were horizontally planted in the pots.
- After 8 days of planting, the sugarcanesets kept in greenhouse conditions germinated.
- In addition, untreated setts were planted and kept in a greenhouse.

3.2 dsRNA-treated and untreated sugarcane at the germination stage using reverse transcription PCR (RT-PCR).

3.2.1 RNAisolation

- The miRNeasy mini kit (Qiagen) was usedto isolate total RNA and a small RNA fraction enriched with miRNAs from frozen tissues according to themanufacturer's instructions.
- For disruption and homogenization, 50 mgof frozen tissues were put immediately into 700 l QIAzol lysis reagent in a clean microfuge tube.
- The homogenate tubes were placed on the benchtop for 5 minutes at room temperature (15–25°C).
- 140 µl chloroform was added to the homogenate tubes, which were then securely capped and forcefully stirred for 15 seconds.
- The homogenate tubes were placed on the benchtop at room temperature for 2–3 minutes.
- At 4°C, samples were centrifuged for 15 minutes at 12,000 rpm.
- The top aqueous phase was moved to a new collecting tube, and 525 l of 100% ethanol was added and pipetted up and down many times to fully mix it.
- The samples were pipette led into aRNeasy Mini spin column in a 2 ml collection tube, including any precipitate that may have developed, and centrifuged at 10,000 rpm for 15 sec at room temperature (15–25°C) and flow-throughwas collected. For complete RNA purification, RNeasy Mini spin columnswere saved.

3.2.1.1 Small RNA (sRNA)-enriched fractionpurification

- For sRNA-enriched fraction purification,

450 µl of 100 percent ethanol was added to the collected flow-through from the previous step and well mixed by vortexing.

- The materials were pipetted into a 2 ml collection tube containing an RNeasy MinElute spin column. The lid was gently closed, and the flow-through was discarded after centrifugation for 15 seconds at 10,000 rpm at room temperature (15–25°C).
- The RNeasy MinElute spin column received 700 µl buffers RWT. To wash the column, the lid was gently closed and centrifuged for 15 seconds at 10,000 rpm. The flow-through was discarded.
- In the RNeasy MinElute spin column, 500 µl of buffer RPE was pipetted up. The lid was gently closed and centrifuged at 10,000 rpm for 15 seconds.
- The RNeasy MinElute spin column was filled with 500 µl of 80 percent ethanol. To dry the spin column membrane, the lid was gently closed and centrifuged for 2 minutes at 10,000 rpm. The collection tube and the flow-through were discarded.
- A new 2 ml collection tube was filled with the RNeasy MinElute spin column. The lid was opened and centrifuged at 10,000 rpm for 5 minutes.

- In a 1.5 ml collection tube, the RNeasy MinElute spin column was inserted, and14 µl RNase-free water was piped over thespin column membrane. To elude the sRNA-enriched fraction, the lid was gentlyclosed and centrifuged for 1 minute at 10,000 rpm.

3.2.2 TotalRNAextraction

TotalRNAwaspurifiedfromsugarcaneleavesasdescribed

3.2.3 cDNAsynthesis

The procedure described was used to makefirst and second strand cDNA.

3.2.4 PCR a mplification of Coatproteinregion of *SCSMV*

All of the PCR amplifications were done in a thermocycler (model Thermocycler Pro, Eppendorf, USA) with the appropriate primers.

Forwardprimer:5′GCGCCCATGGAACACAACCCGGATTGAAC3′

Reverseprimer:5′GCGCGCGCCTCATCTTCCCTACGCAGGTC3′

ReactionMixture

Reactioncomponents	Volume(µl)
SynthesizedcDNA	1.0
10XAdvantage®2PCRBuffer	2.5
dNTPs(10mM)	1.0
ForwardPrimer	1.0
ReversePrimer	1.0
Advantage®2PolymeraseMix	1.0
RNase-freeWater	17.5
Total	25.0

⇒ **PCRprogram:**

95°Cfor5min35cycles of

94°C for 1 min,,60°C for 45 sec
72°C for1min 72°for5min

4°for ever

3.3 Referencegeneidentification

To measure the viral titer, the best reference gene was necessary for normalizing the expression values during the quantitative PCR experiment. Thus, sixteen candidate reference genes were chosen to validate the best reference gene among healthy and SCSMV

infected sugarcane.

3.3.1 Candidate reference gene PCRamplification

All of the PCR amplifications were done in a thermocycler (model Thermocycler Pro, Eppendorf, USA) with the appropriate primers.

ReactionMixture

Reactioncomponents	Volume(µl)
SynthesizedcDNA	1.0
10XAdvantage®2PCRBuffer	2.5
dNTPs(10mM)	1.0
ForwardPrimer	1.0
ReversePrimer	1.0
Advantage®2PolymeraseMix	1.0
RNase-freeWater	17.5
Total	25.0

⇒ **PCRprogram:**

95°Cfor5min35cycles of
94°C for 1 min50°C for 45 sec
72°C for1min
72°for5min4°for ever

Table1: Primerdetailsforcandidatereferencegene

Sugarcane Referenc e genename	Gene accession number	Primer Sequence (5' - 3')	
		Forward primer	Reverse primer
Sc18SrRNA	TC121827	GTGACGGGTGACGGAGAATT	GACACTAACGCGCCCGGTAT
Sc25SrRNA	TC136324	GCAGCCAAGCGTTCATAGC	CCTATTGGTGGGTGAACAATCC
Sc28SrRNA	TC136324	CCTGATCTTCTGTGAAGGGTTCGA	GGTTCGATTAGTCTTTCGCCCTA
ScTUBA	TC127622	TTTGCCCGTGGTCACTACA	GCGTTGAAGACGAGGAAGCCC
ScTUBB	TC132925	CAAGGAGGTGGACGAGCAGATG	GACTTGACGTTGTTGGGGATCCA

ScACTIN	TC120930	GTATTGTTCTCGACTCTGGTGATGG	TCTCAGGTGGTGCAACGACC
ScELF1A	TC115797	CAGTGCTGGACTGCCACA	CTCCACCACCATGGGCTT
ScELF4A	TC144572	TTGTGCTGGATGAAGCTGATG	GGAAGAAGCTGGAAGATATCATAG
ScUBQ	TC115741	ACCACTTCGACCGCCACTACTG	CACCACCTAGCAAGGCTTTCCATTT
ScGAPDH	TC152171	CACGGCCACTGGAAGCA	TCCTCAGGGTTCCTGATGCC
ScAPRT	TC137307	TGACACATTTCCCAACCTCAA	ATCTCTCTCCCTGAGTGGCA
ScCYP	TC147806	CGGACTTCATGTGCCAGGGC	CGTTGGTGTTGGGCCCGGCG
ScP4H	TC125479	GCGACATCAGAACAGTGTGAA	TTGTACTCTCCGCGGTTTCT
ScCUL	TC 152731	TGCTGAATGTGTTGAGCAGC	TTGTCGCGCTCCAAGTAGTC
ScCAC	TC123574	ACAACGTCAGGCAAAGCAAA	AGATCAACTCCACCTCTGCG
ScPRR	TC837693	GCCAAATTCAGGCAGAAAAG	CACCCTAGGCCTTGTTTCAG

3.3.2 QuantitativePCR(qPCR)

The following procedures and settings were used to accomplish quantitative PCR (qPCR)amplification using an MX3005P therrmocycler (stratagene/ Agilent Technologies).

Reaction Mixture

Reactioncomponents	**Volume(µl)**
Reverse transcribed cDNA	1
RealQ-PCR 2X mastermix (Amplicon)	10
Forward primer	0.4
Reverse Primer	0.4
RNase-free water	8.2
Total	

⇒ **PCRprogram:**

95°Cfor5min40cycles of

94°C for 1 min52°C for 45 sec

72°C for1min

3.3.3 MeltingcurveandPCRefficiency

- TheRT reactionmixwithoutreversetranscriptaseservedasanegativecontrol.

- Forinternalcontrol, primers NtActin_FP:5′-ATGGCAGACGGTGAGGATATTCA- 3′andNtActin_RP: 5′-GCCTTTGCAATCCACATCTGTTG-3′ wereusedforamplification.

- After completing PCR cycling, dissociation curves were constructed at 95°C to test PCR specificity.
- The comparative cycle threshold was used to calculate the quantities of RNA accumulation levels as RQ values.(C_T)($2^{-\Delta\Delta Ct}$)method.
  $2^{-\Delta\Delta C_T}$=Normalizedexpressionratio
- Determine the difference between the test sample's CT and the calibrator's CT,socalled$\Delta\Delta C_T$:

$$\Delta\Delta CT = \Delta C_T(test) - \Delta C_T(calibrator), (2)$$

- For both the test sample and the calibrator sample, standardise the CT of GOI to that of ref. ThisisΔC_T:

$$\Delta C_T(test) = C_T(target,test) - C_T(ref,test) \Delta C_T(calibrator) = C_T(target,calibrator) - C_T(ref,calibrator), (3)$$

- Prior to quantitative analysis, the quality of the amplification, dissociation, and standard curve were examined using 10-1 to 10-5 dilutions of cDNA template and MxPro software (version1.0; Stratagene/Agilent Technologies) to determine the specificity of detection and efficiency (RT products of mature miRNA and the internal control).
- The dissociation curve / melt curve is created by gradually increasing the temperature from 55°C to 95°C while monitoring the intensity of fluorescence. A single sharp peak at your unique amplicon's melting temperature (Tm) should be obtained.
- Three technical replicates were used for allqPCR reactions.
- For each set of primers, standard curves were created using a 10-fold serial dilution of the cDNA template to calculate the

gene specific PCR efficiency.

- The standard curve was used to calculate the slope value and correlation coefficient.

3.4 ValidationofReferencegene

Four distinct web-based visual basic appletswere used to confirm the reference gene, including

→ delta-Ctmethod
→ NormFinder
→ GeNorm
→ BestKeeper.

An additional resource The Rank of eachreference gene was also calculated usingRefFinder.

The Ct values were converted to Q values (relative quantities) using the delta Ct method,as follows:

$$Q = E^{\Delta Ct} \quad (4)$$

Where,

E= efficiencyofreferencegene

ΔCt=Ct values differ between the test(infected) and control (healthy) samples.

3.4.1 Web-basedvisualbasicapplets

- These four visual basic applets, Genorm, NormFinder, and QFinder, included relative amounts or Q values.
- The Q values in GeNorm and NormFinder were used to calculate the stability measure (M) and stability ranking.
- Average Ct values were used to evaluatein BestKeeper and RefFinder.
- For each of the 16 reference genes, thestandard deviation (SD), coefficient of variance (CV), and Pearson correlation values (r value) were determined.

- The reference gene/genes with the lowest CV and SD were deemed the most stable reference gene/genes.

3.5 Calculation ofvirustiter

The MX3005P thermocycler (Stratagene/Agilent technologies) was used for all qPCR stages. For each PCR cycle, a standard curve was constructed by plotting the value of Ct against the amount of cDNA in each of the three standard-dilution duplicates using linear regression analysis.

The following formula was used to calculate PCR efficiency:

$$E=e^{\ln 10/-s}-1 \quad (5)$$

where,

Slope(s)=-3.3(E=2)representsa100%efficiency.

- For both the virus and the endogenous reference gene, Average Ct (Ctav) was calculated for the three technical replicatesfor each sample, as follows:

$$Ct_{av}=(Ct_1+Ct_2+Ct_3)/3 \quad (6)$$

WhereCt_1,Ct_2,Ct_3arethe threevaluesofthethethree technicalreplicates.

- The ΔCt technique (Rasmussen, 2001) wasused to calculate the relative quantity of virus in each sample:

$$\Delta Ct_{av}=[Ct_{av}(ref)-Ct_{av}(Virus)] \quad (7)$$

Where*ref*isthereferencegeneusedasreferencei.e. *Sc18SrRNA*

- The ΔΔCtav value was calculated to compare the amounts of viral titer presentin different samples:

$$\Delta\Delta Ct_{av}=\Delta Ct_{av}sample1-\Delta Ct_{av}sample2 \quad (8)$$

- The viral genome's fold change (FC)between different samples was calculated as follows:

  $FC=2^{-\Delta\Delta Ct_{Av}}$(9)

4. RESULT AND DISCUSSION

4.1 RT-PCR product analysis on a 1% Agarose/EtBr gel of treated and untreated sugarcane plants

Tenellado et al. (2003) were the first to demonstrate that dsRNA produced by a bacterial production system can provide virus resistance to plants. Many researchers have since conducted anti-viral research in this field. In comparison to previous virusresistance techniques, RNA-mediated viral resistance is more effective and biosafe. The dsRNA produced by this RNAi approach accumulates in the environment, and these dsRNAs are persistent in vivo for many days after inoculation, even when the plant is wet. Until now, viral resistance research in plants has been limited to a few functional genes of a few viruses.

We used the coat protein gene of SCSMV and RT-PCR analysis to show that RNAi is triggered by externally supplied dsRNA in thisinvestigation. Sugarcane sets of healthy andmosaic symptomatic plants were gathered and immersed for 3 hours in 2-liter distilled water containing crude dsRNA extract. The bacterial crude extract contained about 500g of dsRNA. These setts were then planted in pots and kept in a greenhouse environment. Total RNA was extracted from the leaves of healthy, dsRNA- treated, and untreated plants after 60 days of growth (tillering stage) and reverse transcribedto cDNA. SCSMV was found by employing SCSMV_CP specific primers to amplify theCP gene.

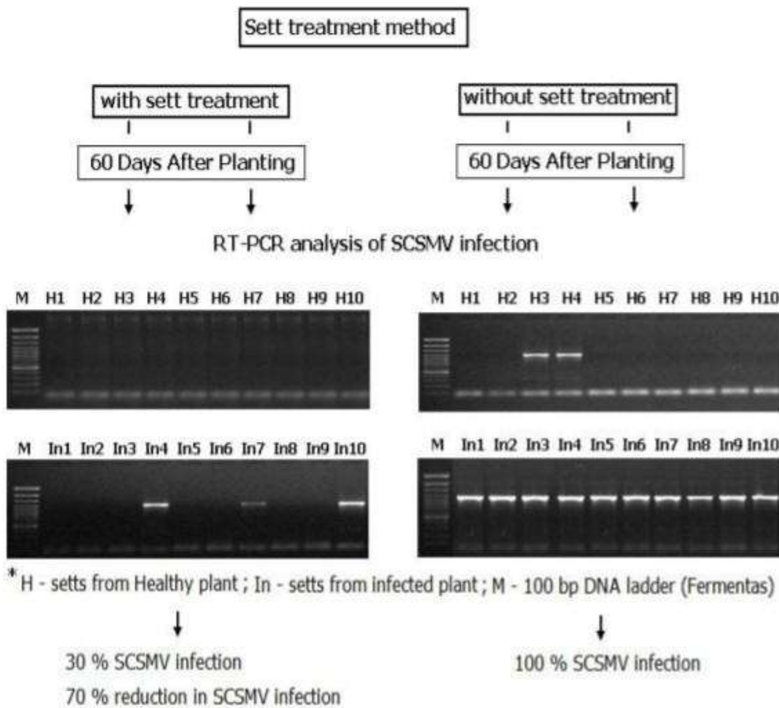


Figure 1:Electrophoresis of the RT-PCR amplified product of the CP gene from healthy,dsRNAtreated, and untreated sugarcane leaf samples on a 1% Agarose/EtBr gel.

4.2 SCSMV virus titre determinationrequires the discovery of a reference gene

There was no virus detection in healthysugarcane samples before and after treatment, however in infected sugarcane samples, 70-80% of the plants revealed no virus detection in treated plants and 100% frequency of virus infection in untreated plants (Figure 1). The fact that dsRNA-treated plants have a lower rate of viral infection indicates that RNAi has spread throughout the plant. In vitro, the dsRNAs remained stable. Cymbidium mosaic virus (CymMV) expression was also 40-fold lower in CymMV CP dsRNA-treated plants than in its other counterpart, indicating a significant reduction of virus in orchid plants. When virus encoded dsRNA was sprayed on the leaf of plants, SCMV infection in maize, PVY infection in tobacco, and CLcuRV infection in cotton were all reported.

4.2.1 Characterization of a potential gene inhealthy sugarcane and sugarcane with mosaic symptoms

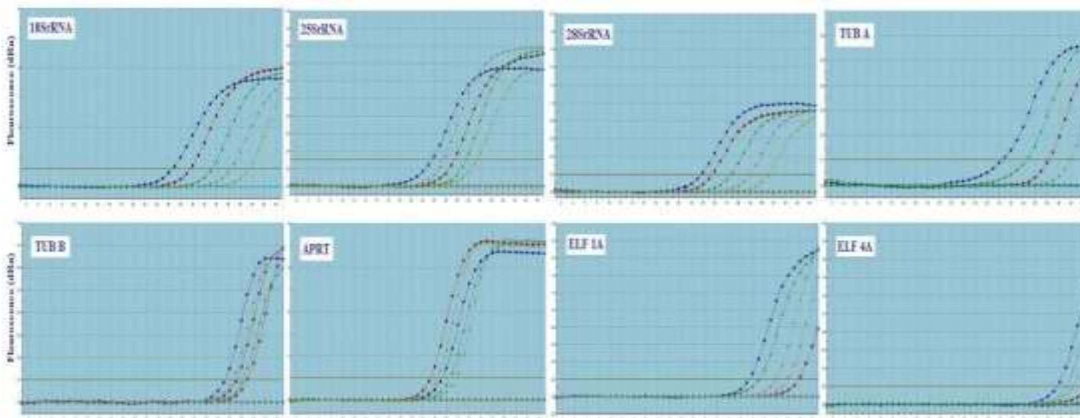
In gene expression analysis investigations, the reference gene is crucial. Previously, referencegenes with a specialized involvement in cellular processes or ribosomal RNA encoding genes, such as the ACTIN gene, were employed to normalize measurement of gene expression levels. The expression of reference genes fluctuates depending on the stress level of the plants. As a result, finding an appropriate reference gene that expresses consistently in both normal and stressful conditions is critical. Iskander et al., (2004) proposed using GAPDH and 25SrRNA for sugarcane normalization. Sugarcane TUB A and GAPDH are indicated in drought stress, GAPDH and ELF 1A in salt stress, and 25SrRNA in Ustilago scrianinea infection. Previous research on virus-infected plants revealed that 18SrRNA and TUB B were

involved in BVDY infection in rice, and ELF 1A was involved in RSBDV infection in wheat.

Sixteen candidate genes were chosen to find a viable potential reference gene for healthy and mosaic virus symptomatic sugarcane. Each primer pair was evaluated for Real-Time PCR amplifications that resulted in the presence of a single product of predicted size, as determined by melting curve and Agarose/EtBr gel (1%) electrophoresis. Table2 lists the primer sequences, PCR efficiency, and regression coefficient (R2) for each candidate gene. Three biological replicates of each sample were tested, the Ct values

obtained from these were pooled, and a mean was computed, as stated in the procedures. ScTUB A has a PCR efficiency of 84, while Sc25SrRNA has a PCR efficiency of 108.9.For each candidate gene, a standard curve was created using serial 10-fold dilution, which was confirmed by the association between Ct values and dilution-fold (Figure 2).

The mean Ct value and standard deviation (SD) of 16 candidate genes range from 25.48to 38.75 and 0.26 to 2.18, respectively, as shown in Table 2. Sc18SrRNA had the highest level of expression (mean Ct SD = 25.48 0.26) followed by Sc25SrRNA (mean Ct SD = 26.510.31) and ScELF 4A (mean CtSD = 38.75.98).



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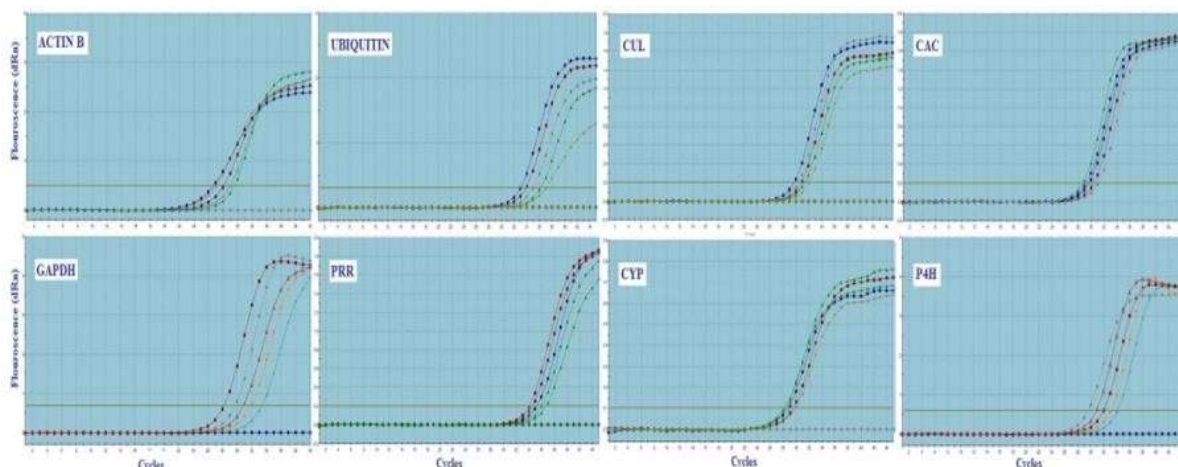


Figure 2: Candidate gene PCR amplification plot utilizing cDNA from healthy sugarcane leaves

This is in a 10-fold dilution series (50 ng/l, 5 ng/l, 0.5 ng/l, 0.05 ng/l, and 0.005 ng/l of template).Triplicates of two biological replicates were used in the reactions.

Table 2: Analyzed candidategenes

Gene	Melting temperature(°c)	PCR efficiency (%)	Mean Ct	R^2 value
Sc18srRNA	85.7	103.00	26.68	0.999
Sc25srRNA	87.1	110.18	25.66	0.998
Sc28srRNA	86.3	108.90	25.67	0.982
ScTUBA	85.8	84.00	26.55	1.000
ScACTIN	86.0	107.13	26.78	0.992
ScTUBB	86.5	105.60	35.26	0.970
ScELF1A	81.3	98.76	33.49	0.998
ScELF4A	75.8	101.30	39.43	0.989
ScUBQ	81.5	104.83	33.05	0.999
ScGAPDH	81.8	99.00	28.24	1.000
ScAPRT	80.3	107.40	25.80	0.986
ScCYP	80.8	103.00	28.10	0.976
ScP4H	81.3	102.83	29.63	0.987
ScCUL	78.8	106.14	29.78	0.995
ScCAC	78.2	88.54	28.75	0.997
ScPRR	81.3	109.17	34.00	0.998

4.2.2 Selection of the most stable referencegene

Because choosing a reference gene is such an important task for differential expression analysis, it should be carefully considered. Normally, reference genes are thought to have steady expression; however, they are subject to considerable variations in stability during

biotic and abiotic stressors. To analyses expression stability, numerous statistical algorithms were developed, including geNorm, NormFinder, BestKeeper, the delta-Ct approach, and the RefFinder WEB-based software. NormFinder and BestKeeper were employed among the visual basic applets, while GeNorm and the delta-Ct results came from RefFinder, a web-based application that

incorporates the most commonly used statistical techniques.

In this study, fifteen candidate reference genes were examined in healthy and mosaic symptomatic sugarcane to find the optimal reference gene. To choose the most stable reference gene in mosaic symptomatic illness conditioned sugarcane, four different visual basic applets were used to examine expression stability of potential reference genes: NormFinder, BestKeeper, RefFinder, and delta-Ct. The raw Ct-values were converted into relative expression values and then entered into NormFinder and GeNorm, while the raw Ct-values were used to analyse the data in the RefFinder tool. The results are presented in the form of a stability value (SV), with the lowest stability indicating the most stable reference gene and vice versa.

4.2.3 Analyses using GeNorm andNormFinder

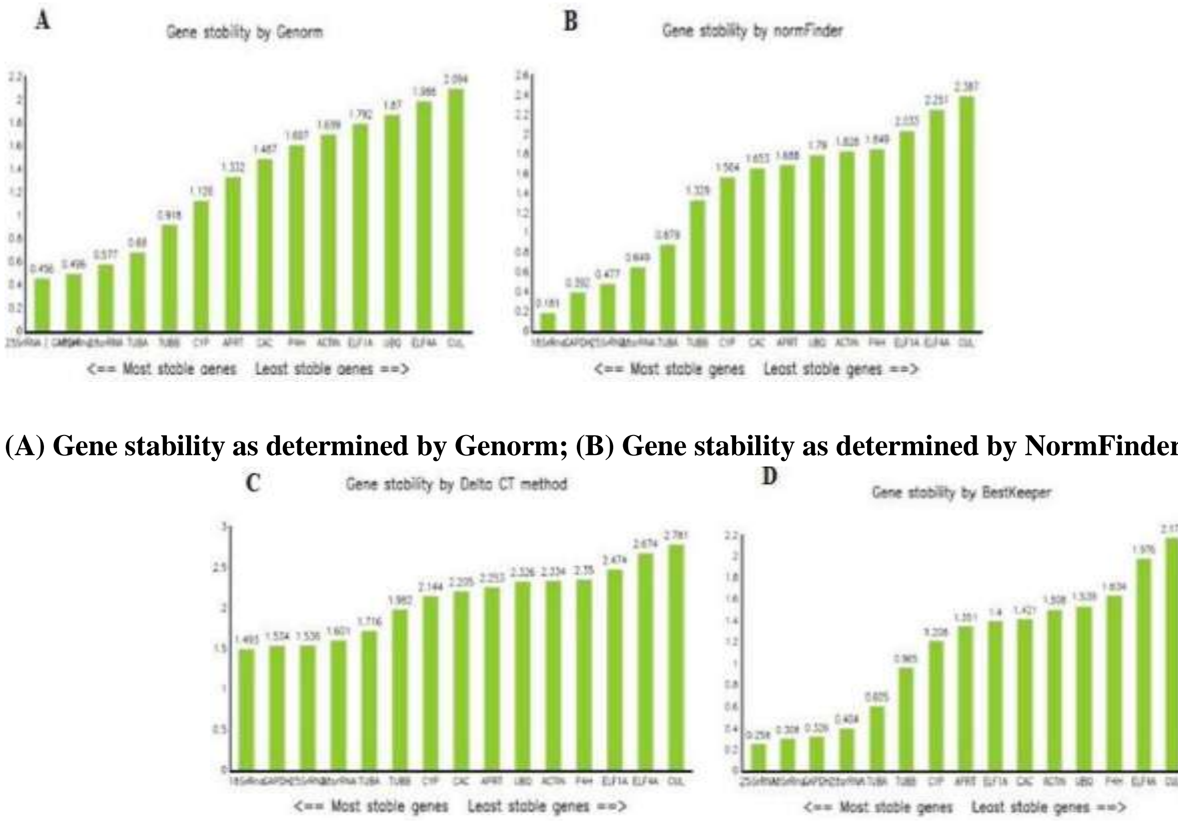
Table shows the results. The most stable expressed gene provided by GeNorm and

NormFinder was 18SrRNA (SV = 0.496; SV = 0.181), followed by 28SrRNA (SV = 0.577) and GAPDH (SV = 0.392). In healthy and mosaic virus symptomatic sugarcane, the combination of 25SrRNA and GAPDH (SV = 0.456) was also ranked as the most stable reference gene for RT-PCR normalization. (See Figures 3 a and 3 b.)

To normalize gene expression in the sugarcanesamples stated above, 18SrRNA, GAPDH, and25SrRNA are sufficient.

4.2.4 Analyses using Delta Ct andBestKeeper

Generally, the Delta Ct and BestKeeper rankings of the most stable reference gene revealed the same order. The most stable reference genes were 18SrRNA (SV = 1.49;SV = 0.258) and GAPDH (SV = 1.53; SV = 0.308), followed by 25SrRNA, 28SrRNA, TUB A, TUB B, CYP,CAC,APRT, UBQ, ACTIN,P4H,ELF 1A, ELF 4A, AND CUL,
according to the two visual basic applets. (See Table 3; Figures 3c and 3d.)



(A) Gene stability as determined by Genorm; (B) Gene stability as determined by NormFinder

y the Delta Ct technique; (D) Gene stability as determined byBestKeeper

ession stability and ranking in healthy and virus-infectedsugarcane

The Y-axis shows the stability value, which was calculated using four distinct visual basic applets;

on stability rankings of sugarcane potential reference genes; from most stableto least stable, the stability values are listed

ank	NormFinder		GeNorm		deltaCt		BestKeeper	
	Gene	bility	Gene	bility	Gene	bility	Gene	bility
1	3SrRNA	0.172	5rRNA	0.343	5rRNA	0.62	18SrRNA	0.21
2	Sc25SrRNA	0.208	25srRNA	0.343	25srRNA	0.63	25srRNA	0.58
3	ScTUBA	0.229	APRT	0.464	TUBA	0.63	APRT	0.71
4	ScP4H	0.230	P4H	0.657	CUL	0.66	28SrRNA	0.73
5	ScAPRT	0.240	TUBA	0.726	CAC	0.69	GAPDH	0.79
6	ScCYP	0.245	CYP	0.777	CYP	0.74	ACTIN	0.81
7	ScUBQ	0.254	UBQ	0.827	ELF4A	0.76	TUBA	0.85
8	ScACTIN	0.265	ACTIN	0.876	ACTIN	0.85	TUBB	0.89
9	Sc28SrRNA	0.290	28srRNA	0.955	28srRNA	0.86	CAC	0.91

10	ScTUBB	0.348	TUBB	1.023	TUBB	0.93	CUL	0.98
11	ScCUL	0.384	CUL	1.102	P4H	0.95	UBQ	1.08
12	ScCAC	0.503	CAC	1.172	APRT	0.99	ELF1A	1.43
13	ScELF1A	0.557	ELF1A	1.274	ELF1A	1.05	ELF4A	1.87
14	ScELF4A	0.575	ELF4A	1.456	UBQ	1.6	PRR	1.89
15	ScPRR	0.649	PRR	1.56	PRR	1.76	P4H	1.96
16	Sc25SrRNA And ScGAPDH	0.136						

4.2.5 Analyzed with RefFinder

RefFinder is a web-based tool that produces results that are comparable to the four programmes discussed above. Sc18SrRNA and Sc28SrRNA were the most stable reference genes, according to RefFinder, NormFinder, and GeNorm analyses, followed by ScGAPDH. BestKeeper and Delta-Ct analyses agreed with NormFinder and GeNorm, indicating that Sc18SrRNA is the most stable gene, followed by ScGAPDH. RefFinder ranks Sc18SrRNA

and ScGAPDH as the most stable referencegenes as a consequence of the four algorithms utilised (Table 4). We also discovered that a combination of Sc25SrRNA and ScGAPDH might be used as a possible reference for normalising expression analyses (Figure 3).

ScCUL and ScACTIN, on the other hand, were the least stable candidate reference genes in this investigation. In contrast to earlier findings, ScELF 1A expression was lowest.

e 4: Delta Ct, BestKeeper, NormFinder, and GeNorm were used to rank each candidategene, and RefFinder was used to provide a comprehensive ranking.

Method	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
DeltaCT	8SrRNA	GAPDH	25SrRNA	28srRNA	TUBA	TUBB	CYP	CAC	APRT	UBQ	ACTIN	P4H	ELF1A	ELF4A	CUL
BestKeeper	8SrRNA	GAPDH	25SrRNA	28srRNA	TUBA	TUBB	CYP	APRT	ELF1A	CAC	ACTIN	UBQ	P4H	ELF4A	CUL
Normfinder	8SrRNA	GAPDH	25SrRNA	28srRNA	TUBB	CYP	TUBA	UBQ	ELF1A	APRT	ACTIN	ELF4A	P4H	CAC	CUL
Genorm	25SrRNA|GAPDH	18SrRNA	28srRNA	TUBA	TUBB	CYP	APRT	CAC	P4H	ACTIN	ELF1A	UBQ	ELF4A	CUL	

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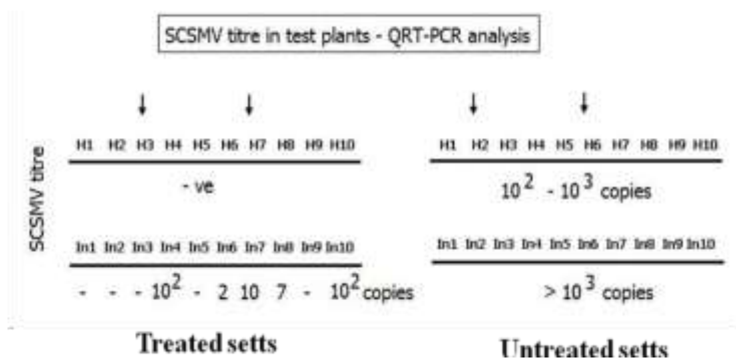
Recommended comprehensive ranking	5rRna	GA PDH	25S rR NA	28S rR NA	T U B A	T U B B	CYP	APR T	CAC	UBQ	EL F1A	A C TI N	P4H	E LF4 A	C U L

4.2.6 Calculation of virus titers

The enzyme-linked immune sorbent assay is commonly used to calculate virus titer, although this method can also be used to generate an approximate estimate of virus titer. Aside from that, viral titers are calculated using quantitative northern blot analysis. The qPCR method for calculating virus titer, on the other hand, is a reliable method that has been successfully used to quantify virus titer for retroviruses, adenoviruses, and herpes simplex virus.

The relative viral titer was estimated in this investigation using Sc18SrRNA as a reference gene, which was chosen since it was the most stable, as described in the previous section.The virus titer of SCSMV encoded dsRNA treated and untreated sugarcane differed significantly. SCSMV _CP specific dsRNA treated sugarcane plants had a viral titer of lessthan 102 copies during the tillering stage, whereas untreated sugarcane had a titer of more than 103 copies.The dsRNA-treated healthy sugarcane plants had no virus titer, but the dsRNA-untreated healthy sugarcane plants had 102-103 virus copies.



ane treated with dsRNA and sugarcane not treated withdsRNA

The titer of SCSMV in treated plants ranges from 2 to 6 copies at the tillering stage, whereas the entire incidence of SCSMV infection in untreated plantlets is 100%

5. CONCLUSION

Sugarcane setts were taken from healthy and SCSMV-infected plants and treated with a crude extract of SCSMV_CP specific dsRNA (500g/ml). The setts, both treated and untreated, were planted in pots and allowed to grow in a greenhouse.There was a requirementto determine the most stable reference gene in

healthy and SCSMV infected sugarcane plants in order to calculate viral titer in dsRNA treated and untreated sugarcane plants. Four alternative analytical methodologies were used to evaluate sixteen putative reference genes based on the stability of transcript profiles across healthy and SCSMV-infected sugarcane. During virus infection, stability assessments of gene expression identified Sc18SrRNA and Sc28SrRNA as the most stable reference genes; however, acombination of Sc25SrRNA and ScGAPDHcan also be employed as a reference gene.

Using 18SrRNA as a reference gene, virus titers were estimated in dsRNA-treated and untreated sugarcane plants, revealing that virus copies in dsRNA-treated sugarcane plants ranged from 2 to 6 copies, while virus copies in untreated sugarcane plants exceeded 103 copies.

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