7-26-2011

Population Genetics of Cell to Cell Movement of Wheat Streak Mosaic Virus

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POPULATION GENETICS OF CELL TO CELL MOVEMENT OF WHEAT STREAK MOSAIC VIRUS

by

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A THESIS

Presented to the Faculty of
The Graduate College at the University of Nebraska
In Partial Fulfillment of Requirements
For the Degree of Master of Science

Major: Biological Sciences

Under the Supervision of Professors Roy French
and T. Jack Morris

Lincoln, Nebraska
August, 2011

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University of Nebraska, 2011

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Previous workers in the lab created an infectious clone of *Wheat Streak Mosaic Virus* (WSMV) designated S1RN. An additional infectious clone with a GUS insert was also created to permit easy observation of virus movement (Gus1RN). A single point mutation made within the HC-Pro region created a mutant of WSMV designated PS81 that was unable to cause a systemic viral infection, although it could infect small clusters of cells on inoculated leaves. A similar mutation was made in the Gus1RN clone and it was designated Gus46. These particular mutants were capable of reverting back to wild type WSMV at high frequency and able to cause a systemic infection. We used this system to assess the multiplicity of infection (MOI) of the virus. MOI was calculated using the PS81 mutant as a control and the Gus46 as the experimental virus used to track virus movement. Wheat plants were inoculated with either PS81 or Gus46 and systemic leaves were collected at 20 and 28 days post inoculation (dpi), respectively. No significant difference was determined in the reversion rate between Gus46 and PS81. The reversion rate of Gus46 was determined and the number of virus genomes with a specific substitution at a particular location per cell was estimated to calculate the MOI of WSMV. The MOI for WSMV was determined experimentally to be approximately 11 genomes. This data supports our hypothesis that only a very limited number of virions escape and move from cell-to-cell during plant virus infections.

The low MOI observed is a severe bottlenecking event for a virus population. This causes a low genetic diversity within the virus population. Narrow genetic bottlenecking during cell-to-cell movement causes a higher selective pressure on viruses and enables viruses to quickly select for adaptive genomes. This ability to select for adaptive genome might offset the negative outcome of bottlenecking, i.e. the loss of fitness by enabling a plant RNA virus to rapidly respond to environmental changes. Further research is necessary to fully understand and appreciate the role MOI has on virus population genetics.

Acknowledgements:

I thank all those who have supported and helped me with this research. To my family and friends who kept giving me encouragement until till I could see the light at the end of the tunnel. A big thank you to my committee members, Dr. Roy French, Dr. Jack Morris and Dr. Satyanarayana Tatineni for all their assistance and expertise. A special thanks to Dr. Roy French for teaching me the basics in plant virology and guiding me throughout this research. I also want to acknowledge all the people who have given me great input and advice along the way.

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CHAPTER 1

POPULATION GENETICS OF CELL TO CELL MOVEMENT OF WHEAT STREAK MOSAIC VIRUS

INTRODUCTION

Wheat Streak Mosaic Virus (WSMV) is a severe pathogen of wheat (*Triticum aestivum L.*) first reported in 1932 (McKinney, 1937). WSMV can be found in all major wheat growing regions of the world (Brakke, 1971). In the Great Plains region of the United States, WSMV is estimated to cause about 5% yield losses annually, but 100% localized yield losses are not unusual (French and Stenger, 2003; 2001; Lu *et al.*, 2011). WSMV has a fairly wide host range within the Poaceae family. The virus infects all varieties of wheat and some isolates can also infect barley, oats, rye, wild grasses, and various varieties of maize and millets (Brakke, 1971; French and Stenger, 2003).

WSMV is transmitted in a semipersistant manner by the wheat curl mite, an eriophyid mite (*Aceria tosichella* Keifer formerly identified as *Aceria tulipae* Keifer) (Slykuis, 1955; Brakke, 1971; Orlob, 1966; Amrine and Stansy, 1994). Wheat curl mites do not have wings and depend entirely on wind for their movement. Both the nymphal stages and adults of the wheat curl mite can transmit the virus, but only nymphs may acquire it (Slykhuis, 1995; del Rosario and Sill, 1965). A feeding time of at least fifteen minutes is needed for a nymph to acquire the virus from an infected plant (Wegulo *et al.*, 2008). Wheat curl mites remain vectors of the virus for almost their entire lives (normally two to four weeks depending on temperatures), although the transmission efficiency of adult mites decreases with age (Wegulo *et al.*, 2008). Green living plant tissue is necessary for the survival of mites, without a food source they will perish within days. Mites cannot vertically transmit WSMV to their progeny through the egg stage of

their life cycle. Each mite must, acquire the virus from an infected plant (Slykhuis, 1955; Staples and Allington, 1956; Orlob, 1966; Amrine and Stansy, 1994).

Low rates of seed transmission of WSMV in maize have been observed (Hill *et al.*, 1974). Researchers from Australia discovered that seed transmission of WSMV in wheat occurs at a rate of 0.5 to 1.5% in eight different genotypes of wheat (Jones *et al.*, 2005). This low level seed transmission would have little impact on the disease cycle here in the United States because both the virus and the wheat curl mite are already established within all growing regions of wheat (Wegulo *et al.*, 2008).

The disease cycle of WSMV begins with the presence of over-summering hosts (corn, grasses and volunteer wheat) that permit the wheat curl mites to survive and reproduce over the summer. The over-summering hosts allow the transmission of WSMV from an infected winter wheat crop the previous season to be carried over to the next season's crop. The mite is able to increase its population throughout the summer and transmit the virus to the newly emerging wheat in the fall. In the Great Plains region, volunteer wheat that emerges before harvest of the summer host creates a "green bridge" for the mites. This permits the mites to shift from their over-summering host to the volunteer wheat, and survive on them until the new wheat crop begins to sprout (Wegulo *et al.*, 2008). Once winter wheat begins to emerge, the mites can transmit WSMV to the seedlings, where the virus will survive over the winter. For this reason, it is import to control volunteer wheat in regions where WSMV is a problem. With the removal of volunteer wheat, the disease cycle can be broken.

Wheat plants infected with WSMV typically display stunting and chlorotic streaks in a mosaic pattern on their leaves these symptoms become more prevalent as

temperatures increase (Wegulo *et al.,* 2008). Infected plants often develop sterile heads causing severe yield losses. Severe symptoms are seen when wheat plants are infected young, thus leading to severe stunting, reduced tillering, poor seed set, and lower seed weight (Brakke, 1971, 1987; Edwards and McMullen, 1987).

WSMV is the type species of the genus *Tritimovirus* within the family *Potyviridae* (French and Stenger, 2003; Stenger *et al.,* 1998). *Potyviridae* is the largest and most economically important family among plant viruses (Shukla *et al.,* 1994). Formerly, WSMV was classified as a member of the genus *Rymovirus*, along with other eriophyid mite-transmitted potyviruses (Zugula *et al.,* 1992; Stenger *et al.,* 1998; Salm *et al.,*1996). Phylogenetic analysis demonstrated that this vector-based classification was paraphyletic. This resulted in the placement of WSMV, along with its sister taxon of *Brome streak mosaic virus*, in a new genus *Tritimovirus* (Stenger *et al.,* 1998).

Particles of WSMV are flexuous rod-shaped, approximately 15 nanometers (nm) in diameter and 700 nm in length (Brakke, 1971). WSMV is a positive sense single-stranded RNA virus, consisting of 9,384 nucleotides (nt), excluding the poly A tail. It is translated as a single polyprotein that is cleaved into at least 10 mature proteins when processed by three viral proteinases (P1, HC-Pro and NIa) (Stenger *et al.,* 1998; French and Stenger, 2003). The WSMV strain used in this research was Sidney 81, a representative of an American WSMV isolate, recovered near Sidney, Nebraska in 1981 (Brakke *et al*., 1990).

Several functions of potyviral proteins have been researched to identify their role in the virus disease cycle. P1 functions as a proteinase that cleaves between itself and helper component-proteinase (HC-Pro) (Verchot *et al.,* 1991; Choi *et al.,* 2002). P1 is

required for efficient genome amplification of potyviruses (Verchot and Carrington, 1995). Several functions have been defined for the HC-Pro protein. HC-Pro plays a major role in effective transmission of the virus by aphids and wheat curl mite vectors to host plants (Atreya *et al.,* 1992; Stenger *et al.,* 2006). This protein also functions as a *cis*-acting protease that processes the polyprotein (Carrington *et al.,* 1989; Oh and Carrington, 1989). Additionally, HC-Pro appears to be involved in several other stages of the virus disease cycle, including disease synergism (Pruss *et al.,* 1997; Shi *et al.,* 1997), replication (Kasschau and Carrington, 1995; Klein *et al.,* 1994; Kasschau *et al.,* 1997), long-distance, cell-to-cell movement (Cronin *et al.,* 1995; Atrey and Pirone, 1993; Syller, 2006; Rojas *et al.,* 1997; Kasschau *et al.,* 1997), and suppression of post-transcriptional gene silencing (Kasschau and Carrington, 1998; Anandalakshmi *et al.,* 1998). The P3 protein has been shown to be a key determinant of pathogenicity and virulence in several potyviruses (Sáenz *et al.,* 2000; Johansen *et al.,* 2001; Hjulsager *et al.,* 2002; Jenner et al., 2003; Suehiro *et al.,* 2004). The specific biochemical function of P3 in WSMV is still unknown, although, research suggests an involvement in the replication process (Choi *et al.,* 2005). Cylindrical inclusion body protein (CI) has ribonucleotide hydrolysis and RNA helicase activities (essential for virus RNA replication) (Fernandez *et al.,* 1995, 1997; Lain *et al.,* 1990, 1991). In addition, CI is critical for both long-distance and cell-to-cell movement within the plant (Carrington *et al.,* 1998). 6K1 and 6K2 are short proteins encoded in the genome and their function is unknown. The third virus-encoded proteinase is nuclear inclusion protein a (NIa) which serves as the VPg, a viral protein covalently bound to the 5'-terminus of genomic RNA (Murphy *et al.,* 1990; Shahabuddin *et al.,* 1988). Nuclear inclusion protein b (NIb) is

assumed to be the viral RNA-dependent RNA polymerase because of the presence of conserved polymerase motifs (Domier *et al.,* 1986; Lain *et al.,* 1989). The coat protein (CP) is a structural protein and it is required for aphid transmission (Atreya *et al.,* 1991; Atreya *et al.,* 1995; Gal-On *et al.,* 1992). CP is essential for both cell-to-cell and long-distance movement within infected plants (Dolja *et al.,* 1995; Rojas *et al.,* 1997). These proteins make up the genomes of most potyviruses functioning in viral virulence and the disease cycle.

RNA viruses must complete multiple steps to cause a systemic infection within a plant. First, the virus gains entry into a plant cell through wounds and begins replication. Second, the newly formed virus must travel into adjacent cells through plasmodesmata and replicates in the newly invaded cells. Third, the virus must then access the phloem of the plant to move long-distances within the same leaf and between organs. Lastly, the virus must exit the phloem and initiate replication and cell-to-cell movement in young tissues distant from the initial infection site (Cronin *et al.,* 1995; Carrington *et al,* 1996). All of the above steps are necessary for a systemic viral infection to occur within a plant.

RNA viruses have a high mutation rate. This is due to RNA-dependent RNA polymerase having an error rate of about 10^{-4} per nucleotide per replication in addition to having no capability of proof-reading the product (Harrison, 2002). Hence, in a 10,000 nt RNA virus genome like that of WSMV, one mutation per viral genome would occur with every replication cycle (García-Arenal *et al.,* 2003). Thus in 10,000 nt genome, the probability of one mutation occurring at a particular site would be 0.01%. If it is assumed that there are approximately 10^6 viral genomes per infected cell (French and Stenger, 2003), then the probability of a mutation occurring at a particular site within a

genome (0.0001) would result in about 100 genomes having a mutation at the particular site of interest. In order to estimate the frequency of a specific substitution at a specific location, one must consider that a mutation could be any one of three different nucleotides. From this one can deduce that within every WSMV infected cell there would be approximately 33 genomes with a specific nucleotide substitution at every location.

In studying the evolution of viruses and plant resistance, a simple model to consider is that a single point mutation could be sufficient for an avirulent virus to become virulent. In the above equations we showed that there are approximately 33 copies of an exact nucleotide substitution within every infected cell. In the WSMV model we are studying here, it was shown previously that a single nucleotide change at nucleotide 1341 from Adenine to Guanine in the mutant PS81 was sufficient to alter the phenotype from a localized infection to a systemic wild type infection. This model demonstrates that virulent viruses can be produced that are able to overcome plant resistance and move systemically throughout the plant.

The objective of this research was to measure the rate at which an avirulent WSMV mutant, with a single point mutation, is able to revert to a virulent wild type WSMV and cause a systemic infection (Stenger *et al.,* 2006). The second objective was to calculate the multiplicity of infection (MOI) of WSMV. This simple model shows that each infected cell has several copies of the exact mutation needed to become a systemically moving virus. Therefore, we hypothesized that avirulent viruses rarely move from cell-to-cell and over long distances within an infected plant.

MATERIALS AND METHODS

Plasmid Constructs

A WSMV cDNA infectious clone in a low copy number plasmid pACYC-177 (pACYC-WSMV) was previously used to make the following plasmid constructs; PS81-46-1, Gus-46-1-1, S1RN and Gus1RN (Figure 1 and Figure 2) (Choi *et al.,* 1999). The infectious clone was constructed from Sidney 81, which has been completely sequenced and can be found in GenBank as accession no. AF057533 (Stenger *et al.*, 1998). The S1RN is a wild type WSMV infectious clone. The Gus1RN is the S1RN construct with a GUS coding sequence positioned between P1 and HC-Pro (Choi *et al*., 2002). PS81-46-1 was produced by the amplification of the HC-Pro coding region using *Taq* DNA polymerase in PCR to generate a single nucleotide substitution (Stenger *et al.,* 2006). PS81-46-1 has a non-synonymous point mutation at nucleotide 1341 located within the HC-Pro region altering Adenine to Guanine and causing an amino acid switch from Glutamic Acid to Glycine (Stenger *et al.,* 2006). Gus-46-1-1 is the same PS81-46-1 construct with a GUS coding sequence inserted between P1 and HC-Pro (Figure 2) (Stenger *et al.,* 2006). The GUS coding sequence was incorporated into the infectious clone to allow for the monitoring of virus movement within a wheat plant using the GUS assay.

Four plasmid constructs were used for this research. S1RN as previously described is the wild type WSMV. It was used as a positive control for when studying the reversion of PS81 to wild type. Gus1RN is wild type WSMV, with GUS sequence inserted. It served as the positive control for the reversion experiments using Gus46.

Gus1RN allowed the movement of wild type WSMV to be observed within the inoculated leaves. Both Gus46 and PS81 have a single point mutation within the HC-Pro region which inhibits systemic movement unless a reversion event takes place. The Gus46 construct permitted the detection of cell-to-cell movement within an inoculated leaf, without having to use two differently tagged viruses. PS81 served as a control to ensure the addition of the GUS sequence in Gus46 did not have an adverse affect on the number of viruses which reverted back to wild type WSMV and moved systemically throughout the wheat plant.

Transformation

All stock plasmid constructs PS81-46-1, Gus-46-1-1, S1RN and Gus1RN were diluted with sterile water. All four plasmids were transformed to *E. coli* competent cells (JM109) via Plasmid Quick Kit manufactured by Promega, Madison, WI. For each of the transformations 100 µl and 10 µl were plated on 2-YT agar plates and incubated at 37˚C for 12 to 14 hours. A single small white colony from each plasmid transformation plate was transferred using a sterile toothpick to a 500 ml flask containing 200 ml of YTA broth and 200 µl of 50 mg/ml Carbenicillin antibiotic. Flasks were placed in an incubator with a shaker set at 300 rpm and 37˚C for 14 to 16 hours.

Plasmid Isolations

Perfectprep Plasmid Maxi Kit manufactured by Eppendorf, Westbury, NY was used to isolate the plasmids from the *E. coli* cells as per the manufacturer's protocol.

Except that 300 µl of sterile water was used instead of 500 µl to elute plasmid DNA, since the plasmids were a low copy number. Plasmids were stored at -20˚C.

Restriction Nuclease of Plasmids

Each of the four plasmids was digested with enzyme *Sal* 1 for one hour at 37˚C. The digestion mixture consisted of 5 µl of plasmid, 0.5 µl of *Sal* 1 enzyme (Promega in Madison, WI), 1 µl of Buffer D (Promega in Madison, WI) and 3.5 µl of sterile water. Digested plasmids were electrophoresed in 1% agarose gels with 4 µl of 1% ethidium bromide. Ten µl of digested plasmid plus 2 µl of loading dye was loaded into the gel with a 1 kb DNA ladder. The gels were electrophoresed for one hour at 140 Volts. Plasmids were also digested with *Sac* 1 to check for correct fragment sizes. Five µl of digested plasmid was added to 0.5 µl of *Sac* 1 enzyme (Roche in Indianapolis, IN), 1 µl of Buffer J (Roche in Indianapolis, IN) and 3.5 µl of sterile water. The mixture was digested for one hour at 37˚C and electrophoresed as above.

Plasmid concentration was measured using a spectrophotometer. Samples were diluted 1:50 by adding 5 µl of plasmid to 245 µl of sterile water. The concentration of each plasmid in µg per µl was calculated using the absorbance value obtained at 260 Abs. The absorbance values were multiplied by the dilution factor (50) and divided by 20 to give the plasmid's concentration.

Plasmids were linearized with the *Not* 1 enzyme (BioLabs in New England) and Buffer #3 (BioLabs in New England). Thirty µg of each plasmid was used to make RNA transcripts. Three µl of *Not* 1 enzyme and 50 µl of Buffer #3 were added to each digestion, sterile water was used to bring the total volume up to 500 µl. Digestions were

placed at 37˚C for two hours. Gel electrophoresis was performed using a 1% agarose gel loaded with 10 µl of 1 kb ladder (DNA Molecular Marker X, Roche), 8 µl of plasmid digestions plus 2 µl of loading dye for each sample and electrophoresed for one hour at 140 Volts.

Plasmid Precipitation

Phenol extractions were completed on each plasmid by adding 25 µl of 0.5 EDTA to the 500 µl digested plasmid. A 500 µl of phenol was added to each plasmid and vortexed for 20 seconds. The mixture was then centrifuged at 13,200 rpm for 5 minutes and collected the supernatant in a new tube. To reprecipitate DNA from the supernatant 50 µl of 3M NaOAc and 500 µl of 2-propanol (Isopropanol) were added. Tubes were held on ice for 20 minutes and then centrifuged for 10 minutes at 13,200 rpm. The supernatant was discarded from all the tubes and the pellet was washed with 70% EtOH. The 70% EtOH was discarded and the tubes were placed in a drier to evaporate remaining EtOH. Plasmids were resuspended in 60 µl sterile water and placed in the freezer for long term storage at 4˚C.

Transcriptions

SP6 MEGAscript, High Yield Transcription Kit produced by Ambion in Austin, TX was utilized in generating RNA transcripts for wheat plant inoculations at the second leaf stage. To prepare a transcript the following was added to a 1.5 ml microfuge tube; 8 µl of nuclease-free water, 4 µl of 10x reaction buffer, 4 µl of 50 mM ATP solution, 4 µl of 50 mM CTP solution, 4 µl of 10 mM GTP solution, 4 µl of 50 mM UTP solution, 4 µl

of m^7G(5')ppp(5')G Cap Analog (100 A 254 U per 137 µl concentration), 4 µl of DNA Template concentration was 0.5 µg/µl (DNA digested with Not I) and 4 µl of Enzyme Mix giving a total volume of 40 µl. When a lower concentration of cap analog was used (0.4 U/ µl), only 4 µl of nuclease-free water was added instead of 8 µl and 8 µl of analog was added to the mix instead of 4 µl to ensure the same concentration of analog was in the transcription mixture. The mixture was incubated for 5 1/2 to 6 hours at 37˚C.

Wheat Plants

Tomahawk wheat seeds from 2007 and 2008 were planted in six inch diameter clay pots with approximately 16 to 18 seeds per pot in a pre-mixed sterilized soil. Plants were grown in a greenhouse and watered daily. All wheat plants used in this experiment were grown in this manner.

Inoculation of Wheat Plants

RNA transcripts were inoculated onto wheat plants at 7 to 9 days old depending on the emergence of the second leaf. The inoculum was prepared by adding; 110 µl of sterile water, 150 µl of 2% Na Pyrophosphate, and 30 µl of Bentonite. All inocula were maintained on ice during preparation. Each pot had approximately 16 to 18 wheat plants that were inoculated by sprinkling carborundum (abrasive powder) onto the leaves and rubbing on the inoculum. Long sterile q-tips were used to paint the inoculation mixture onto each leaf and inoculum was rubbed into the leaves using gloved finger tips. Inoculated leaves were rinsed with water to wash off the buffer from inoculated leaves.

GUS Assay

GUS assay was completed at 4 days post inoculation (dpi) for plants inoculated with Gus46 and Gus1RN. The primary inoculated leaf (1st wheat leaf) was used for these assays as described by Choi *et al.* (2000). Leaves collected from the inoculated wheat plant were cut into 1 cm segments. Leaf segments were placed into a 10 ml round bottom tube. Five ml of GUS solution (8.9 ml of sterile water, 1 ml of 0.5mM $NaPO^4$, 10 µl of Tween 20 and 200 µl of 100mM X-gluc (0.05 g of X-glu dissolved in 960 ml DMSO)) was added to each tube. Vacuum infiltration was applied 3 to 4 times for 5 minutes until leaves looked soaked and appeared darker in color. Tubes were placed in an incubator shaker overnight at 37˚C and 200 rpm. The following day the solution was removed and the tubes were filled with 100% EtOH and incubated for 1 hour at room temperature. Washing with 100% EtOH was repeated until leaves were no longer dark green. Leaves were stored in 70% EtOH in the refrigerator for future analysis.

Calculating the Number of Wheat Cells Infected per Inoculated Leaf

Using the inoculated leaves (1st wheat leaf) stained with GUS, the infected area of each leaf was measured. This was done using a Zeiss SteREO Discovery.V12 microscope with an AxioCamMRC5 camera connected to a computer. The AxioVision Rel. 4.8.1 (11-2009) program was used to take pictures and evaluate the area of each GUS stained spot. The average area of all the GUS stained infected areas were calculated using an Excel spreadsheet. After obtaining the average infected area of an inoculated leaf the average number of infected cells per inoculated leaf was determined. Previous research on the 1st leaf of a wheat plant was utilized to determine the average number of

wheat cells infected per inoculated leaf. In 1982, Jellings and Leech established that there are typically 0.00124 mesophyll cells per μm^2 in the 1st leaf of a wheat plant. Using the average leaf area infected, multiplied by the number of mesophyll cells (0.00124) per unit area (μm^2) provided an estimate of the average number of cells infected with virus per inoculated leaves.

RNA Isolation from Systemic Leaves

At 20 dpi, leaves were collected from plants infected with wild type S1RN and the mutant PS81. Two systemic leaves were removed from each plant to be tested for the presence of WSMV. At 28 dpi two systemically leaves were collected from plants inoculated with Gus1RN and Gus46, respectively. Leaves from each plant were placed in a tissue grinding bag for RNA extraction. Leaves were stored in a ziplock bag inside at 4˚C until RNA was extracted.

RNA was extracted from the collected systemic leaves as follows. Samples were placed in a grinding bag with 1 ml of sterile water and hand ground until the leaves became slightly translucent. For each sample, 250 µl of leaf extract was transferred to a fresh 1.5 ml tube, with 250 µl of 2x Glycine buffer (pH 9.0), 25 µl of 20% SDS and 500 µl of phenol:Chloroform:isoamyl alcohol. The samples were mixed and centrifuged for 5 minutes at 13,200 rpm. Approximately, 300 µl of supernatant was transferred to a fresh 1.5 ml tube on ice. To the extraction tubes, 30 µl of 3M NaOAC and 900 µl of 100 % EtOH was added and inverted to mix the contents. Samples were centrifuged in a cold centrifuge for 5 minutes at 13,200 rpm. The pellet was washed with 500 µl of 70% EtOH. Tubes were placed upside down for 5 to 10 minutes to allow the EtOH to

evaporate. To ensure all the EtOH had been removed, tubes were transferred to a vacuum drier for 5 minutes or until no EtOH remained. Isolated RNA was resuspended with 25 µl of sterile water. All samples were stored at -20˚C.

Primers

Primers homologous to the HC-Pro region were used to identify S1RN and PS81 in systemic leaf samples. The forward primer S81HCF (GENOSYS) 5'GAAATGCACACATGGACTTAGATGGTAT-3', had a OD of 47.4, 1436.4 µg, 165.9 nmol, Tm= 67.3 degrees C, 30.3 µg/OD, and a MW=8661. The reverse primer S81HCR (GNEOSYS) 5'CATGCTTGTATACTGAGAACAGTCTCTTG-3', had a OD of 64.4, 2072.2 µg, 233.3 nmol, Tm=65.8 degrees C, 32.2 µg/OD, and MW= 8883.

Primers homologous to the coat protein (CP) region of the genome were used to identify Gus1RN and Gus46 in systemic leaf samples. The forward CP primer XV1 (IDT) 5'GATTCCGTTGAAGGATTTGTAACTT-3', had a OD of 24.3 = 100.2, nm = 0.77 mg, Tm= 53.7 degrees C, and MW= 7702.1. The reverse CP primer XC1 (IDT) 5'AACCCACACATAGCTACCAAG-3', had a OD of 21.7 = 103.6 nm = 0.66 mg, Tm= 54.6 degrees C, and MW= 6337.2.

Reverse Transcription

Reverse Transcription was performed on all samples at 42˚C for 1 hour, followed by 99˚C for 5 minutes and held at 10˚C. For one reaction, the reaction mixture for reverse transcription consisted of 11.8 µl of sterile water, 4 µl of 10x buffer (Mg+), 1 µl

of 10 pmole/µl reverse primer, 0.8 µl of 10 mM dNTPs, 0.4 µl of RT enzyme (23 U/µl) and 2 µl of isolated RNA from the systemic leaves.

Taq-PCR

Taq-PCR was performed on all samples. Each reaction mixture for *Taq*-PCR consisted of 36.75 µl of sterile water, 5 µl of 10x buffer (Mg+), 2.5 µl of 10 pmol/µl forwards primer, 2.5 µl of 10 pmol/µl reverse primer, 1 µl of 10 mM dNTPs, 0.25 µl of *Taq* polymerase (5 U/µl), and 2 µl of cDNA, giving a total reaction volume of 50 µl per reaction.

The PCR program used for the S1RN and PS81 samples consisted of 29 cycles. The program had a denaturing temperature of 94˚C for 3 minutes, an annealing temperature of 52˚C for 1 minute and an elongation time of 12 minutes at 72˚C. Samples were held at 4˚C until being removed from the thermocycler and then stored at -20˚C.

The PCR program used for the Gus1RN and Gus46 samples consisted of a denaturing temperature of 95˚C for 2 minutes and 30 seconds. The annealing temperature was 50˚C for 30 seconds and the elongation temperature was 72˚C for 12 minutes. This PCR program consisted of 35 cycles. All samples were held at 25˚C until being removed from the thermocycler and stored at -20˚C.

Agarose Gel Electrophoresis

All *Taq*-PCR products were checked by agarose gel electrophoresis. A 1% agarose gel was made with the addition of 4 µl of 1% ethidium bromide. A 1 Kb DNA Molecular Weight Marker X made by Roche was used as a reference marker (100 µl

DNA molecular marker c 0.07-12.2 Kbp + 100 µl loading dye + 300 µl water = 500 µl 0.5 µg/ 10 µl). For each PCR product, 8 µl of product was mixed with 2 µl of loading dye before loading the gels. All gels were electrophoresed at approximately 140 volts for 50 to 60 minutes.

PCR Product Purification

A High Pure PCR Product Purification kit produced by Roche Applied Science (Indianapolis, IN) was used to purify PCR products for sequencing. For PS81, 20 samples positive for reversion back to the wild type WSMV were chosen to be sequenced. Additionally, 20 Gus46 samples positive for systemic infection of WSMV with the GUS insert were also sequenced. Since Gus46 positives were detected using CP primers, these samples were processed again using HC-Pro primers. This allowed the HC-Pro region to be amplified during PCR and to be used for sequencing in order to confirm a true reversion of Guanine back to Adenine in the HC-Pro region had taken place in the Gus46 samples (Stenger *et al.*, 2006).

For PCR product purification 58 µl of sterile water was added to each 42 µl of PCR sample to bring the total volume of product up to 100 µl. PCR products were purified using a High-Pure spin column kit (Roche). The 100 µl of PCR product was transferred to a 1.5 ml tube, 500 µl of binding buffer was added and mixed thoroughly. This mixture was transferred to the upper reservoir of a High pure filter tube inside a collection tube. The tubes were centrifuged for 30-60 seconds at 13,200 rpm. The flow through solution was discarded and the filter tube was placed back into the same collection tube. A 500 µl of wash buffer was placed in the reservoir of each filter tube

and centrifuged for 1 minute at 13,200 rpm. The flow through solution was discarded. Washing was done twice with 200 µl of wash buffer and centrifuged for 1 minute at 13,200 rpm. The flow through solution and the collection tubes were discarded. Each filter tube was transferred to a fresh 1.5 ml tube. A 50 µl of elution buffer was added to the reservoir and centrifuged for 1 minute at 13,200 rpm. The fresh 1.5 ml tubes contained the purified PCR product, which was stored at -15 to -25˚C. Purified PCR products were electrophoresed in a 1% agarose gel at 140 volts for one hour to assess its quality prior to sequencing.

Sequencing

Purified PCR samples were quantified with a spectrophotometer. Sequencing required a 20 µl sample with a concentration of 2 ng/µl per 100 nucleotides. PS81 samples were diluted to a concentration of 24 ng/µl because the PS81 PCR product was approximately 1,200 nt long. In contrast, Gus46 samples which were diluted to a concentration of 60 ng/µl, since the Gus46 PCR product was approximately 3,000 nt long. The primers used for sequencing of the HC-Pro region were made by Integrated DNA Technologies in Coralville, IA. The forward HC-Pro primer's sequence was 5'-CACTGAAGCCGAAATGCACACA-3', which targeted nucleotides 1149 to 1170 on the strand being sequenced. A reverse HC-Pro primer 5'-TGGCGTGCCTCAACCTCTTCAT-3' and targeted nucleotides 1498 to 1467 on the strand being sequenced. All samples were sent to Davis Sequencing in Davis, CA for sequencing.

Calculating the Number of Virus Genomes per Gram of Plant Tissue

Previous quantitative results determined by Brakke *et al.* (1968) and Tatineni *et al.* (2010) allowed the number of genomes of WSMV per gram of wheat tissue to be calculated. Both of these earlier experiments determined the number of WSMV genomes within systemically infected wheat leaves. Brakke's measurement at ~12 dpi was 7 µg WSMV/g tissue = 3.5 µg WSMV RNA/g tissue = 7×10^{10} genomes of WSMV per 1 gram of plant tissue (Brakke *et al.*, 1968). Tatineni's paper in 2010 established the number of WSMV genomes within infected wheat plants at 14 and 28 dpi, using Real Time PCR (Tatineni *et al.*, 2010). Using the weight in grams of plant tissue used for each Real Time PCR reactions (4.63×10^{-6}) in Tatineni *et al.* (2010), it was possible to calculate the number of WSMV genomes per gram of plant tissue (Figure 12).

Converting Leaf Weight to Leaf Area

To find the average weight of the 1st leaf of a wheat plant, 50 plants were grown in the green house for 11 days. The 1st leaf was removed from wheat plants at 11 days because this was the age of the leaves when samples were taken for GUS assays. Leaves were weighed and the average 1st leaf weight was calculated along with standard deviation and error. Average leaf weight was divided by average leaf area to give the weight (grams) per unit leaf area (μm^2) (Figure 13).

Calculating the Number of WSMV Genomes per Cell

The weight of a wheat cell (grams) was calculated by dividing the weight in grams per unit area taking gram per unit, divided by cells per unit area (Figure 14a).

Genomes per cell were then calculated using genomes per gram of wheat tissue multiplied by grams per wheat cell (Figure 14b).

Calculating the Number of Virus Genomes with a Specific Substitution per Cell

RNA viruses have been shown to have a mutation rate of 10^{-4} per nucleotide per replication (Harrison, 2002). An RNA virus with a genome size of 10,000 nt such as WSMV, would be expected to have one mutation occur per genome during each replication cycle. This is computed by taking 10,000 multiplied by 10^{-4}. The chance of a specific mutation occurring at an exact site within a 10,000 nt genome is calculated at 0.0001 or 0.01%. Hence, there is a 0.01% chance of a mutation taking place at a particular site within a 10,000 nt genome. To establish the chance of a specific mutation happening at an exact site within a genome, the above number is multiplied by 1/3 (Figure 15). To calculate the number of WSMV genomes with an exact mutation within a wheat cell, the results from Figure 14b were multiplied by the chance of a mutation occurring at a particular site (0.0001) and then multiplied by 1/3 (Figure 15). These calculations were done to estimate the number of genomes within a cell with a precise point mutation from Guanine back to Adenine.

Calculating the Number of WSMV Genomes that Move to an Adjacent Cell

Figure 16 shows the equation developed to evaluate the number of WSMV genomes which could theoretically move initially in infected cell clusters. For Figure 17, the Gus46 reversion rate of 0.02120 (Table 7) was placed in part 1, 566 cells (Table 5) was placed in part 2, for part 3 the values from Figure 14 were used at 14 dpi, 28 dpi and

Brakke's and for part 4 the values from Figure 15 were used at 14 dpi, 28 dpi and Brakke's. Figure 17 was used to solve for X (the number of WSMV genomes that move out of the infected clusters of cells) the MOI.

RESULTS

Plasmid Digestion

Controls for these experiments included wild type S1RN and the wild type with the GUS insert (Gus1RN). Two transformants were isolated for each plasmid construct. Purified plasmids were digested with enzymes *Sac* 1 and *Sal* 1 to ensure the plasmids generated WSMV cDNA without any rearrangement of sequence. All the transformed plasmids were digested with *Sac* 1 and analyzed by gel electrophoresis for the three fragments of expected size as follows: S1RN fragments of 5,108 nt, 4,283 nt and 3,712 nt (Figure 3 lanes 2-3); Gus1RN fragments of 6,908 nt, 4,283 nt and 3,712 nt (Figure 3 lanes 4-5). The same plasmids were also digested with *Sal* 1 to create a linearized S1RN fragment of about 13,100 nt (Figure 4, lanes 2-3). Two fragments of expected sizes, one large band of ~13,100 nt with one smaller band of ~1,800, were seen with Gus1RN (Figure 4, lanes 4-5).

For PS81, 4 transformants were selected and purified. Similarly, 16 transformants were obtained for Gus46. Both PS81 and Gus46 plasmids were digested with enzymes *Sac* 1 and *Sal* 1 to ensure the plasmids transformed correctly. Both S1RN and Gus1RN were used as the positive controls for the correct fragment sizes when testing PS81 and Gus46 for accurate transformations. Fragment sizes of *Sac* 1 digested were 6,908 nt, 4,283 nt and 3,712 nt, which is the same as S1RN (Figure 5, lanes 4-5). When PS81 was digested with *Sal* 1 it produced a single fragment of ~13,100 nt, which is identical to S1RN (Figure 5, lanes 8-9). These digestions confirmed the transformants were properly transformed.

When Gus46 was digested with *Sac* 1 the same three fragment sizes produced by Gus1RN were observed (Figure 5, lanes 2-3). The expected fragment sizes were also displayed when Gus46 was digested with *Sal* 1 ~13,100 nt and ~1,800 nt (Figure 5, lanes 6-7). All transformants digested were compared to the corresponding controls and if fragment sizes were not identical to the positive controls, transformants were discarded.

For this research, only one transformant of S1RN (1), Gus1RN (1) and PS81 (1) were used in making RNA transcripts for wheat plant inoculations. For Gus46, three transformants were used; Gus46 (2), Gus46 (3) and Gus46 (16). The concentrations of all the plasmid preparations were measured and can be seen in Table 1. A DNA concentration above 0.22 µg/ µl was used for making RNA transcripts. Plasmid preparations with concentrations below 0.22 µg/ µl were discarded. To produce a linear plasmid with a cDNA concentration of 30 µg/µl, each transformant was digested with *Not* 1. Figure 6, lane 3 is an example of the correct fragment size for PS81 and S1RN constructs at ~13,100 nt. For Gus46 and Gus1RN constructs, the correct fragment size was ~15,000 nt (Figure 6, lane 2). Once all transformants had been analyzed for accuracy they were used in making RNA transcripts for inoculating 7 to 9 day old wheat plants.

Number of Wheat Cells Infected with WSMV

A GUS assay was performed on the inoculated leaves of 480 wheat plants inoculated with Gus46 transcripts. Of the 480 leaves processed, 68 GUS stained infected areas were observed on the inoculated leaves. Figure 7 shows the visual difference in the GUS staining between a wheat leaf inoculated with wild type Gus1RN (control)

compared to the mutant Gus46. The area of each GUS stained spot corresponds to the area infected with virus. Every GUS stained infected area was measured in μm^2 (Table 2). The average GUS stained area was also calculated per set (Table 3). Each set is comprised of plants which were grown, inoculated and collected at the same time. The average leaf area infected was 451,997 μm^2, with a standard deviation of 632,170 and standard error of 76,662.

We experimentally determined the leaf area infected. This permitted estimating the number of cells infected using the data of Jellings and Leech (1982) which showed that there are 0.00124 mesophyll cells per μm^2 in the 1st wheat leaf (Table 4). This data is presented in sets of plant samples grown, inoculated and collected at the same time for further comparison (Table 5). Taking an average of all 68 GUS stained infected areas. The number of cells infected on inoculated leaves was calculated to be 566 cells, with a standard deviation of 784 and a standard error of 95 (Table 5).

Measuring the Reversion Rate of PS81

Systemic leaves were collected at 20 dpi from 235 wheat plants inoculated with PS81 and 10 wheat plants inoculated with S1RN. S1RN served as a positive control. All 10 wheat plants displayed WSMV symptoms and tested positive for WSMV using *Taq*-PCR. Of the 235 wheat plants inoculated with PS81, 58 were found to be positive for putative reversion back to wild type WSMV (S1RN) based on their ability to move systemically throughout the wheat plant (Table 6). The reversion rate for PS81 was calculated at 0.2468. A positive reversion was expected to produce a PCR product of ~1,200 nt upon gel electrophoresis. Such samples were deemed positive for putative

reversion back to wild type WSMV. This is observed in Figure 8 lanes 3, 5, 6, 7, 8, 11 and 15. *Taq*-PCR products for all PS81 samples tested were electrophoresed on a 1% agarose gel. All positive PS81 gels are not shown.

Measuring the Reversion Rate of Gus46

At 28 dpi, systemic leaves were collected from 184 wheat plants inoculated with Gus46 and 10 wheat plants inoculated with Gus1RN. Gus1RN was used as the positive control. All of the wheat plants inoculated with Gus1RN displayed WSMV symptoms and tested positive for WSMV using *Taq*-PCR. Of the plants infected with Gus46, 39 out of 184 tested positive for putative reversion (Table 7). A reversion rate of 0.2120 for Gus46 mutants was calculated. A single band of ~1,400 bp was produced by the positive control Gus1RN. Gus46 samples were considered positive for reversion if they formed the same size band as the control (Figure 9, lanes 5, 7, 8, 11 and 16). Not all positive Gus46 gels are shown.

Comparison of Gus46 and PS81 Reversion Rate

The ratio of positive revertants within each set of Gus46 and PS81 samples was calculated and statistically compared using a two-tailed non-directional and equal variance student t-test (Table 8). This resulted in a P-value of 0.6999, with 7 degrees of freedom, at a 0.05 level of significance. For the ratio of positive revertants per set for Gus46 samples the standard deviation was 0.1461, with a standard error of 0.0730. For PS81, the ratio of positive revertants per set had a standard deviation of 0.1133 with a standard error of 0.0507.

PCR Analysis for Positive Reversions

Twenty positive revertants were sequenced for each of the PS81 and Gus46 samples analyzed. The positive revertants were purified using a PCR purification kit. The purified PCR samples were electrophoresed to ensure that no PCR product was lost during purification (Figure 10 and Figure 11). A positive reversion for PS81 formed a single ~1,200 nt band. For Gus46 samples, a band of ~3,000 nt was considered positive for reversion (Figure 11). The concentration of positive revertant PS81 and Gus46 samples were determined after their purification (Table 9 and Table 10).

Sequencing Results

The HC-Pro region of 20 samples of PS81 and 20 samples of Gus46 positive for reversion were sequenced. All 20 PCR products of PS81 sequences showed the correct reversion from Guanine back to Adenine resulting in the correct amino acid substitution of Glutamic Acid instead of Glycine. No further mutations were seen within the HC-Pro region close to the point of reversion. For Gus46, 17 out of 20 of the PCR products were sequenced. Three of the Gus46 PRC products were unable to be sequenced. All 17 Gus46 PCR products were true reversions of Guanine back to Adenine. Sequencing results showed no additional mutations near the HC-Pro region.

Calculation of Virus Concentration

The quantitative results obtained by Brakke *et al.* (1968) and Tatineni *et al.* (2010) allowed the number of WSMV genomes per gram of plant tissue to be calculated.

Brakke's data established that the concentration of WSMV was 7 µg/ grams tissue (7 x 10^{10} WSMV genomes per gram of tissue) at ~12 dpi (Brakke *et al.,* 1968). Brakke's data was based on calculating virus yield by density gradient centrifugation. Tatineni obtained slightly different results by examining WSMV infected wheat plants at 14 and 28 dpi using Real Time PCR (Tatineni *et al.,* 2010). Using the weight in grams of tissue used for each Real Time PCR reaction (4.63 x 10^{-6}) (Tatineni *et al.,* 2010), we could calculate the number of WSMV genomes per gram of tissue as shown in Figure 12. At 14 dpi we estimated there were 7.66 x 10^{10} WSMV genomes per gram of tissue and at 28 dpi the number was 2.48 x 10^{10} WSMV genomes per gram of tissue.

Calculation of Leaf Area

The average weight of the 1st leaf of a wheat plant was determined to be 0.0754 grams. A standard deviation of 0.0132 and a standard error of 0.0019 was established using 50 individual 1st wheat leaf samples. The average leaf weight (0.0754 grams per leaf) divided by the average leaf area (451,997 µm^2) was used to calculate a weight per unit leaf area of 0.000000167 grams/µm^2 (Figure 13).

Calculating the Number of WSMV Genomes per Cell

To find the number of WSMV genomes per cell the number of grams of virus per wheat cell was calculated. The weight in grams of wheat leaf tissue per unit leaf area (0.000000167 grams/µm^2), divided by the number of wheat cells per unit leaf area (0.00124 cells per µm^2) resulted in a figure of 0.000135 grams per wheat cell (Figure 14a). The number of WSMV genomes per cell was then calculated by taking WSMV

genomes per gram of tissue multiplied by 0.000135 grams per wheat cell (Figure 14b). Tatineni's measurements at 14 dpi resulted in an estimate of 10,341,000 WSMV genomes per cell and approximately 3,348,000 WSMV genomes per cell at 28 dpi. Brakke's measurement resulted in an estimate 9,450,000 WSMV genomes per cell.

Calculating the Number of Virus Genomes with a Specific Substitution per Cell

RNA viruses, like WSMV, have a mutation rate of 10^{-4} per nucleotide per replication (Harrison, 2002; French and Stenger, 2005). WSMV is expected to have one mutation occur per genome assuming this mutation rate and a genome size of 10,000 nt for WSMV. Hence, there is a 0.01% chance of a mutation occurring at a precise site within the WSMV genome. It is therefore expected there is a 0.0033% chance of a specific mutation occurring at an exact site within the WSMV genome (Figure 15). As shown in Figure 15 the number of WSMV genomes with a precise mutation within an infected wheat cell could then be estimated to be approximately 345 copies at 14 dpi and 112 copies at 28 dpi using Tatineni's measurements and approximately 315 copies using Brakke's measurement.

Estimating the Number of WSMV Genomes that Move to an Adjacent Cell

A model was created (Figure 16), to calculate the number of WSMV genomes that move to an adjacent cell from the initially inoculated cell. First, the reversion rate of Gus46 at 0.2120 is used in part 1 of the equation (Table 7). Second, the number of infected cells per inoculated leaf which was found to be 566 is used in part 2 of the equation (Table 5). Third, the total number of RNA genomes per cell is used in part 3.

These results were estimated to be 10,341,000 WSMV genomes per cell at 14 dpi, 3,348,000 WSMV genomes per cell at 28 dpi and 9,450,000 WSMV genomes per cell using Brakke's measurement. The number of genomes with specific substitution per cell is then used in part 4. It was estimated at approximately 345 genomes at 14 dpi, 112 genomes at 28 dpi and 315 genomes using Brakke's measurement. The previously calculated values were then used in the equation in Figure 16 to solve for X the calculated multiplicity of infection (MOI) of virus in the inoculated leaf. Figure 17 displays the whole equation solving for the number of WSMV genomes that move into adjacent cells (X). The estimated number of WSMV genomes that move to an adjacent cell (MOI) at 14 dpi was 11.23 and 11.20 genomes at 28 dpi. Using Brakke's measurement the estimated MOI was 11.24 WSMV genomes move to an adjacent cell.

DISCUSSION

GUS Assay Used to Determine the Number of Wheat Cells Infected with WSMV

A GUS assay allowed the number of wheat cells initially infected with the mutant Gus46 on the inoculated wheat leaf to be determined. A distinct visual difference was observed between the area infected with wild type Gus1RN and the mutant Gus46 (Figure 7). This visual difference is due to the point mutation from Adenine to Guanine in Gus46, which prevented the mutant virus from moving systemically throughout the plant (Stenger *et al.,* 2006). The point mutation reduced the level of cell-to-cell movement within inoculated leaves (Stenger *et al.,* 2006). This point mutation caused an amino acid change from Glutamic Acid (acidic) to Glycine (neutral). Possibly the change from an acidic amino acid to a neutral one caused a structural change interfering with protein folding. This may give insight into why the virus is unable to move systemically. The restriction in cell-to-cell movement caused by the point mutation allowed the infected area on the 1st leaf to be analyzed and quantified. The average leaf area infected with Gus46 was determined to be 451,997 ± 76,661 μm^2 (Table 2 and Table 3). The number of infected cells per leaf was 566 ± 95.

Systemic Movement within PS81 and Gus46 Inoculated Plants

The optimal times to collect systemic leaves for plants inoculated with PS81 or Gus46 was determined. PS81 was detected systemically at 20 dpi but Gus46 was not detected at this time point (data not shown). Gus46 tended to travel more slowly than PS81 by about 8 days. At 28 dpi, Gus46 could be detected within the systemic leaves.

This difference in rate of systemic movement might be a consequence of Gus46 insert of GUS sequence which increased the size of PS81 by approximately 1,800 nt. It is possible this large genome replicated more slowly and this caused a slight delay in its systemic movement.

Gus46 and PS81 Reversion Rates

The mutant Gus46 and PS81 demonstrated a reversion rate of approximately 0.2120 to 0.2468, respectively. In about 21% to 24% of infections initiated by Gus46 and PS81, the genomes reverted back to wild type and were able to systemically infect the plants. For the revertants to be isolated from the systemic leaves they had to be able to survive the genetic bottlenecking events that occur during cell-to-cell movement.

A comparison of Gus46 and PS81 reversion rates was crucial to ensure that the addition of the GUS sequence in Gus46 did not inhibit the ability of the virus to revert back to wild type or interfere with systemic movement. The results show that there was no significant difference between the reversion rate of Gus46 and PS81 (Table 8). The P-value was calculated to be 0.6999 at a 0.05 level of significance and the null hypothesis was accepted. This proves the reversion rate of Gus46 was not significantly inhibited by the addition of the GUS sequence.

Sequencing of PS81 and Gus46 Positive for Reversion

A sample set of positive reversions of PS81 and Gus46 were sequenced to confirm that true reversion occurred and that no additional random mutation in this region had occurred. A true reversion for Gus46 and PS81 mutants is characterized by an

exchange of Guanine for Adenine at nucleotide 1341 within the HC-Pro protein region. A reversion of Glycine back to Adenine resulted in an amino acid change of Glycine back to Glutamic Acid. These sequencing results showed that true reversions did occur in all Gus46 and PS81 samples that were sequenced. This conformation was vital to ensure that no additional different mutations occurred which could have permitted the virus to travel systemically despite not being an authentic revertant. These results provided certainty that the reversion back to wild type is what permitted the virus to move systemically throughout the wheat plant because no additional mutations were seen in the sequence of the HC-Pro region.

WSMV Genomes per Wheat Cell on the Inoculated Leaf

The quantitative results of Brakke *et al.* (1968) and Tatineni *et al.* (2010) were used to determine the number of WSMV genomes per infected cell. Brakke's and Tatineni's results were used to calculated the number of WSMV genomes per gram of leaf tissue and these numbers were used for further calculations. The average weight of the 1st wheat leaf was established at 0.0754 ± 0.0019 grams. This small standard error demonstrates how consistent the weight is on the 1st leaf of wheat plants. The same is true for the size of the 1st wheat leaf. Several papers by Jellings and Leech addressed the average number of mesophyll cells that compose the 1st wheat leaf to be in an incredibly close range for ~113,026 cells per cm^{-2} to ~133,451 cells per cm^{-2} (Jellings and Leech, 1982, 1984, 1985; Pyke *et al.*, 1990).

The resulting numbers of WSMV genomes per cell calculated from Tatineni's measurement at 14 dpi and Brakke's measurement at 12 dpi were comparatively close.

At 14 dpi we calculated 10,341,000 WSMV genomes per cell, whereas at 12 dpi we calculated 9,450,000 WSMV genomes per cell. By 28 dpi the number of WSMV genomes per cell dropped to 3,348,000. This is not surprising, since plant viruses replicate faster in young expanding plant tissue verses older non-expanding plant tissue (Abdullahi *et al.,* 2001). This fact explains the higher number of WSMV genomes per cell obtained from samples taken at 14 dpi compared to samples collected at 28 dpi. The lower number of WSMV genomes per cell seen at 28 dpi might also be do to the physiology of the wheat plant and its effect on virus replication.

Specific Substitution within the Virus Genome

Using mutant Gus46 enabled us to track the number of viruses which move from cell-to-cell. Gus46 needed to revert back to wild type to move systemically throughout the wheat plant. The calculations showed a 0.01% chance of a mutation occurring at nucleotide position 1341 within the WSMV genome. Hence, there is only a 0.0033% chance of a specific mutation occurring at nucleotide coordinate 1341 within the WSMV genome. The experiment specifically looked for a specific point mutation of Guanine reverting back to Adenine at nucleotide 1341 within the HC-Pro region of the virus. Thus, we were looking for the Gus46 mutant to revert back to wild type WSMV within the wheat plant.

The chance of this event to occur is a percentage (0.0033%) multiplied by the total number of genomes within the cell. The results were 345 copies at 14 dpi and 315 copies at 12 dpi using Brakke's measurement which remained proportional. Whereas, at 28 dpi the resulting number was lower at 112 copies of the exact need mutation for

reversion occurring inside the cell. The number of virus genomes formed with the exact mutation need for reversion to allow a systemic infection is directly comparative to the total number of genomes within the cell.

Number of WSMV Genomes that Move to an Adjacent Cell

The equation (Figure 17) was used to determine the number of WSMV genomes moving into adjacent cells. The equation calculated for the differences in the starting size of the WSMV population within the cell. This is the reason for estimated numbers of WSMV genomes that move from cell-to-cell were similar at 14, 12 and 28 dpi which was approximately 11 genomes. The proportionality of the WSMV population size inside a cell is indicative to the number of viruses within the population that have the chance of reverting back to a wild type WSMV. Consequently, all three calculated values for MOI resulted on the order of 11 WSMV genomes move into an adjacent cell.

Genetic Bottlenecking and its Major Role in Virus Movement

Plant cells force the viruses through a bottlenecking event as they move from cell-to-cell within the plant. Plant tissues are well organized and cells are separated from each other by cell walls connected by plasmodesmata. Ultimately, plant viruses must travel to an adjacent cell through the connecting plasmodesmata or over a longer distance via the phloem. These structural barriers limit the free mobility of the many plant virus genomes in the cell. This is because plant viruses do not lyse the cell and release the entire population of progeny virus to initiate new infections by being able to freely attach to and

enter any cell of the host plant. This restriction in virus movement within a host plant and between cells causes a severe bottlenecking event for virus populations.

The results of this research fully support the idea that plant viruses face severe genetic bottlenecking during cell-to-cell movement. Our data shows that only 11 out of nearly 10 million WSMV genomes located inside an infected cell are able to move into an adjacent cell and initiate a new infection. This explains why reversion of plant virus causing a systemic infection within a plant is an exception and not the norm. Hence, if only 11 genomes are capable of moving into an adjacent cell than the probability of a genome with the ability to travel systemically escaping the cell is tremendously low. These findings supported the hypothesis that avirulent plant viruses are rarely able to escape the inoculated cell and move from cell-to-cell.

Comparison of Result to Previous Research

A paper by González-Jara *et al.* (2009) addressed the multiplicity of infection (MOI) of *Tobacco Mosaic Virus* (TMV), i.e. the number of virus genomes which infect a cell and replicate. Their finding estimated the MOI of TMV to be approximately 6 to 7 (González-Jara *et al.*, 2009). They used TMV tagged with either green fluorescent protein (GPF) or red fluorescent protein (RFP) to approximate the MOI. A significant difference in coinoculated protoplasts was seen meaning their different TMV tags competed with each other. This perhaps distorted their predicted MOI for TMV. Having competition between the two tagged TMV adds another variable such as selective pressure to their results. Competition between their isolates would not permit the true number of viruses moving from cell-to-cell to be calculated efficiently.

In the early part of 2010, Miyashita and Kishino, published a paper addressing the MOI in *Soil-Borne Wheat Mosaic Virus* (SBWMV). For their research they used RNA 2 of SBWMV tagged with two different proteins, yellow fluorescent protein (YFP) and cyan fluorescent protein (CFP). The estimated MOI for SBWMV was 5.97 ± 0.22 in the initially infected cell and 5.02 ± 0.29 in the secondly infected cell (Miyashita and Kishino, 2010). Their results showed separation of the two vectors during infection revealing that competition between the two vectors occurred. Having competition between the two vectors leaves causes more selective pressure than just the basic bottlenecking event for the virus to overcome when trying to move from cell-to-cell. This factor means their result cannot be a true estimate of MOI for SBWMV.

A paper published in late 2010 by Gutiérrez *et al.*, estimated the MOI for *Cauliflower Mosaic Virus* (CaMV). They used two "equi-competitive" CaMV variants tagged with a 40-bp DNA insert to observe the MOI. The predicted MOI for CaMV was determined to be 7.87 ± 2.03 and 6.67 ± 1.43 (Gutiérrez *et al.*, 2010). Even though their results are similar to the two previously described studies they too observed dominance of one variant of CaMV over the other. They addressed this in their discussion saying they cannot explain this observation as the variants used were "equi-competitive". The results of this study along with the other two papers had competition as a variable, which would skew the MOI of the plant virus being studied. Competition adds more selective pressure on the viruses in addition to the bottlenecking event.

It is not surprising that the MOI (11 genomes) for this experiment is higher compared to the three previous studies. Since our strategy for estimating MOI in WSMV did not require a virus tagged with different markers the competition factor was

eliminated. The other three studies concluded variant competition within the host plant skewing their MOI estimates. Our strategy is advantageous because it used a single virus construct with a point mutation and measure reversion back to wild type, which fully restored cell-to-cell movement. The reversion event with the GUS tag allowed the MOI of WSMV to be determined without the complication interference of competition between two individually tagged virus isolates. Sequencing verified that a true reversion back to wild type WSMV did occur and was the authentic factor that allowed the virus to travel systemically throughout the wheat plant.

Importance of MOI of Plant Viruses

Providing experimental approximation of the MOI for plant viruses has a particular important implication on virus populations. MOI levels play a role in virus evolution, selection intensity on genes, genetic exchange, epistatic interactions, hybrid incompatibility, hyperparasitism and the evolution of multipartite and segmented genomes. Having a high MOI favors genetic diversity within the virus population, but slows down virus evolution. An elevated MOI decreases the intensity of selection on each locus since the MOI is the ploidy level of a genome throughout its period of expression. Thus, a low MOI level causes an increase in viral evolution, but a decrease in the amount of genetic diversity within the virus population.

Survival of Virus in Cell-to-cell Movement

The three papers addressed, along with this research, all indicate narrow genetic bottlenecks occurs in cell-to-cell movement within plant RNA viruses that infect

mesophyll cells. Currently the mechanism of the occurrence of genetic bottlenecking during cell-to-cell movement is not known (Miyashita and Kishino, 2010). Further research is needed to address the number of plasmodesmata connecting wheat mesophyll cells to one another to see if the number of plasmodesmata is also playing a role. Previous research on WSMV genetic bottlenecking during cell-to-cell movement suggests this may occur due to sequence homology, via an RNA silencing mechanism (Hall *et al.,* 2001). This would prevent further infection by a virus with sequence homology (Hall *et al.,* 2001).

Since plant viruses face narrow genetic bottlenecking in each cell-to-cell movement it might be deduced that this would result in extinction or very low fitness of the viral population. This might possibly be the end result for an intracellular population traveling from one cell to the next. Nevertheless, due to an abundant number of cells and space for local infection to occur within a host plant, stochastically adaptive viral genomes persist and survival of the viral population as a whole is achieved. In fact it is speculated that plant viruses utilizes bottlenecking events as a fundamental part of their evolutionary mechanism (Miyashita and Kishino, 2010). Narrow genetic bottlenecking during cell-to-cell movement actually helps the virus by quickly isolating adaptive genomes from defective genomes due to the added selective pressure. This may offset the negative outcome of bottlenecking, like the loss of fitness, by enabling a plant RNA virus to rapidly respond to environmental changes.

Future Insight

The experimental procedures used in this research did not depend on a mixed infection of virus to estimate the MOI of WSMV. The predicted MOI for WSMV as measured by reversion of a mutant defective in cell-to-cell movement to wild type was 11 genomes. Estimating the MOI gives plant virologists better insight into the full selective pressures virus populations face within a host plant. The low MOI demonstrates quit clearly that narrow genetic bottleneck is occurring during each cell-to-cell movement event that takes place within the infected wheat plant. With estimated MOI of plant RNA viruses a broader understanding of virus evolution and population genetics within the host plant itself can be obtained. It will also give scientists insight into RNA plant virus survival and epidemiology. Further investigation is needed to fully appreciate the significance of this newly discovered data, but this research gives a foundation to build on for future research and experimentation within plant virology.

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TABLES

Table 1 – Measurements of plasmid concentrations used in making RNA transcripts to inoculate wheat plants.

Plasmid Isolate	**260 Abs**	**Conc. µg/ µl**
S1RN (1)	0.21	0.53
Gus1RN (1)	0.19	0.47
Gus46 (2)	0.09	0.23
Gus46 (3)	0.1	0.24
PS81 (1)	0.13	0.31
Gus46 (16)	0.09	0.23

Table 2 – Area of GUS staining in μm^2 per infection site and the average GUS stained area.

	tube #	leaf	area μm^2		tube #	leaf	area μm^2
Set 3	1	1a	2,277,226	**Set 13**		7b	52,146
	2	2a	105,732		8	8a	1,111,720
	3	3a	75655			8b	209,992
		3b	227182		9	9a	2,075,238
		3c	605,965		10	10b	449,182
	4	4a	95,139			10c	842,604
		4b	192,665		11	11a	83,369
	5	5a	3,812,510		12	12a	356,064
Set 4	1	1a	155,560		13	13a	161,714
	2	2a	576,007			13b	206,738
Set 7	1	1a	325,063		14	14a	100,072
	2	2a	178,207			14b	856,853
		2b	428,365			14c	50,368
		2c	113,644			14d	563,870
	3	3a	161,243			14e	1,084,659
		3b	71,029		15	15a	350,270
	4	4a	75,345		16	16a	131,883
Set 9	1	1a	547,480			16b	936,458
	2	2a	455,987		17	17a	46,078
Set 10	1	1a	23,729			17b	65,405
	2	2a	150,922			17c	26,053
		2b	506,563			17d	167,961
Set 11	1	1a	258,991	**Set 14**	1	1a	213,575
Set 12	1	1a	159,061			1b	850,928
Set 13	1	1a	293,057			1c	598,260
	2	2a	495,869		2	2a	32,269
		2b	375,711		3	3a	315,327
		2c	1,766,223			3b	1,709,225
	3	3c	332,185		4	4a	718,362
	4	4a	155,401		5	5a	86,709
		4b	42,660			5b	120,733
	5	5a	136,425		6	6a	104,292
	6	6a	43,338		7	7a	792,718
	7	7a	147,770		8	8a	201,826
				Average	**area**	**μm^2**	**451,997**

Table 3 – The average GUS stained area of each set of inoculated wheat leaves. The standard deviation and standard error was determined per set. Set 11 and 12 consisted of only one GUS stain and therefore no standard deviation or error could be calculated. The last row is an average of all the GUS staining samples collected.

Sets	Average area µm^2	Standard deviation	Standard error
3	924,009	1,382,158	488,667
4	365,783	297,301	210,224
7	193,271	134,834	50,962
9	501,734	64,696	45,747
10	227,071	250,263	144,489
11	258,991	na	na
12	159,060	na	na
13	428,667	504,438	89,173
14	478,685	487,853	140,831
All sets	451,997	632,170	76,662

Table 4 – The average number of cells infected with Gus46 on the inoculated leave. This was calculated by multiplying the area of the GUS staining in Table 2 by 0.00124 mesophyll cells per μm^2 on the inoculated leaf.

	tube #	leaf	total cells infected per leaf		tube #	leaf	total cells infected per leaf
Set 3	1	1a	2,824	**Set 13**		7b	65
	2	2a	131		8	8a	1,379
	3	3a	94			8b	260
		3b	282		9	9a	2,573
		3c	751		10	10b	557
	4	4a	118			10c	1,045
		4b	239		11	11a	103
	5	5a	4,728		12	12a	442
Set 4	1	1a	193		13	13a	201
	2	2a	714			13b	256
Set 7	1	1a	403		14	14a	124
	2	2a	221			14b	1,062
		2b	531			14c	62
		2c	141			14d	699
	3	3a	200			14e	1,345
		3b	88		15	15a	434
	4	4a	93		16	16a	164
Set 9	1	1a	679			16b	1,161
	2	2a	565		17	17a	57
Set 10	1	1a	29			17b	81
	2	2a	187			17c	32
		2b	628			17d	208
Set 11	1	1a	321	**Set 14**	1	1a	265
Set 12	1	1a	197			1b	1,055
Set 13	1	1a	363			1c	742
	2	2a	615		2	2a	40
		2b	466		3	3a	391
		2c	2,190			3b	2,119
	3	3c	412		4	4a	891
	4	4a	193		5	5a	108
		4b	53			5b	150
	5	5a	169		6	6a	129
	6	6a	54		7	7a	983
	7	7a	183		8	8a	250

Table 5 – The average number of cells infected with Gus46 per inoculated leaf, displayed in sets. The standard deviation and standard error were calculated per set. Set 11 and 12 consisted of only a single GUS stain and therefore no standard deviation or error could be determined. The last row is an average of all the GUS staining samples collected.

Sets	Average cells infected per inoculated leaf	Standard deviation	Standard error
3	1,146	1,714	606
4	454	369	261
7	240	167	63
9	622	80	57
10	282	310	179
11	321	na	na
12	197	na	na
13	532	626	111
14	594	605	175
All sets	566	784	95

Table 6 – The number of plants tested for PS81 that reverted back to wild type WSMV. Positive PS81 was determined using reverse transcription and *Taq*-PCR. Allowing a reversion rate for PS81 to be calculated at 58/235 = 0.2468.

Set	**Number of plants tested**	**Positives by PCR at 20 dpi**
7	48	13
9	46	17
10	46	15
11	50	9
12	45	4
Total	**235**	**58**

Table 7 – The number of plants tested for Gus46 that reverted back to wild type WSMV. Plant positive for reversion were identified using reverse transcription and *Taq*-PCR, giving a reversion rate for Gus46 to be calculated at 39/184= 0.2120.

Set	**Number of plants tested**	**Positives by PCR at 28 dpi**
11	49	2
13B	43	8
15A	48	19
15B	44	10
Total	**184**	**39**

Table 8 – A ratio of the positives within each set of Gus46 and PS81 samples used to calculate the P-value within a student t-test. A t-test with two-tails non-directional, and equal variance with 7 degrees of freedom, at a 0.05 level of significance.

Set	**Gus46**	**Set**	**PS81**
11	0.0408	7	0.2708
13B	0.1860	9	0.3696
15A	0.3958	10	0.3261
15B	0.2273	11	0.1800
Standard deviation	0.1461	12	0.0889
Standard error	0.0730	**Standard deviation**	0.1133
		Standard error	0.0507
P value	0.6999		

Table 9 – Concentration of samples of purified PCR products from systemically infected plants inoculated with PS81. The concentrations were read by a spectrophotometer and the proper dilutions made per the sequencing company requirements.

Set - Sample	260 Abs	Concentration µg/µl
7-10	0.04	0.09
7-30	0.04	0.09
7-44	0.08	0.19
7-46	0.04	0.09
9-8	0.05	0.12
9-11	0.04	0.11
9-24	0.04	0.10
9-38	0.05	0.11
10-4	0.06	0.15
10-9	0.05	0.12
10-20	0.05	0.13
10-26	0.04	0.10
11-44	0.04	0.11
11-45	0.05	0.12
11-47	0.06	0.16
11-48	0.06	0.14
12-25	0.04	0.10
12-32	0.04	0.10
12-33	0.05	0.12
12-40	0.03	0.08

Table 10 – Concentration of samples of purified PCR products from systemically infected plants inoculated with Gus46. The concentration was measured by a spectrophotometer and the proper dilution was made per the sequencing company requirements.

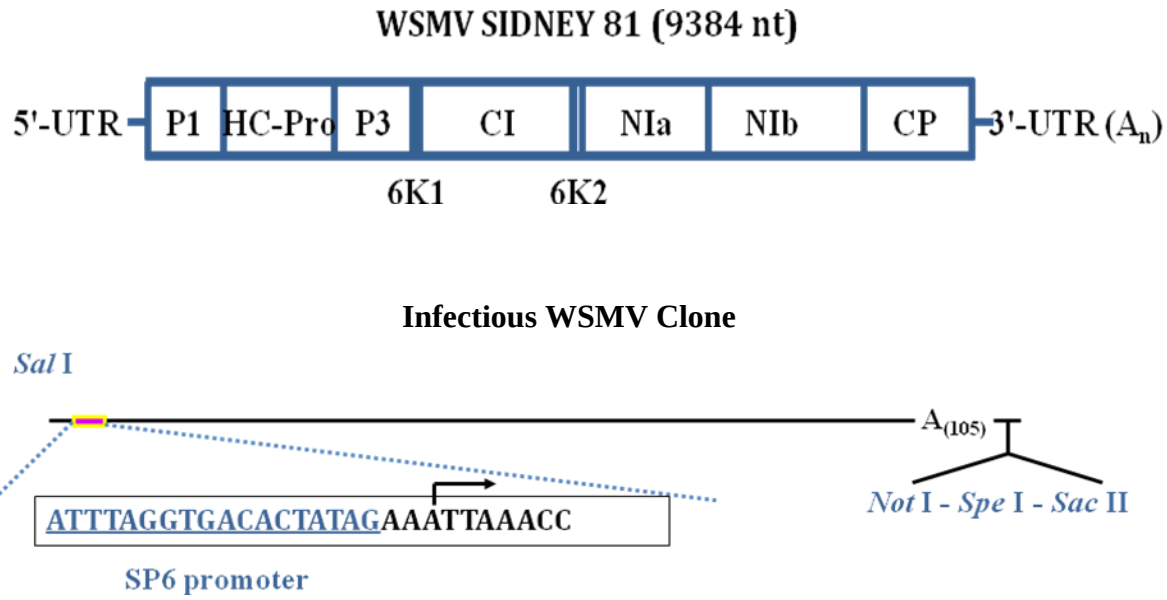
Set - Sample	260 Abs	Concentration µg/µl
11-16	0.03	0.07
13-5	0.03	0.08
13-9	0.04	0.09
15-3	0.03	0.08
15-7	0.05	0.12
15-9	0.05	0.12
15-12	0.05	0.11
15-14	0.07	0.18
15-18	0.07	0.16
15-19	0.06	0.16
15-20	0.05	0.13
15-24	0.05	0.12
15-28	0.06	0.14
15-43	0.05	0.14
15-46	0.05	0.14
15-58	0.05	0.12
15-65	0.05	0.13
15-71	0.06	0.15
15-86	0.06	0.16
15-88	0.06	0.15

Table 11 – The weight of the 1st wheat leaf at 11 days old. The average weight of the 1st leaf was determined along with the standard deviation and standard error within the set of 50 samples.

Wheat Plant	Grams	Wheat Plant	Grams
1	0.0933	26	0.0855
2	0.0839	27	0.0735
3	0.0727	28	0.0861
4	0.0816	29	0.0679
5	0.0771	30	0.0432
6	0.0766	31	0.0726
7	0.0713	32	0.0704
8	0.0857	33	0.0689
9	0.0714	34	0.0707
10	0.0915	35	0.0917
11	0.0821	36	0.0562
12	0.0777	37	0.0628
13	0.0726	38	0.0847
14	0.0792	39	0.074
15	0.0807	40	0.0894
16	0.0476	41	0.065
17	0.1003	42	0.0796
18	0.0626	43	0.0885
19	0.0834	44	0.0749
20	0.0774	45	0.0389
21	0.0934	46	0.0766
22	0.0938	47	0.07
23	0.075	48	0.0755
24	0.0781	49	0.049
25	0.0593	50	0.088
		Average	**0.0754**
		Standard deviation	**0.0132**
		Standard error	**0.0019**

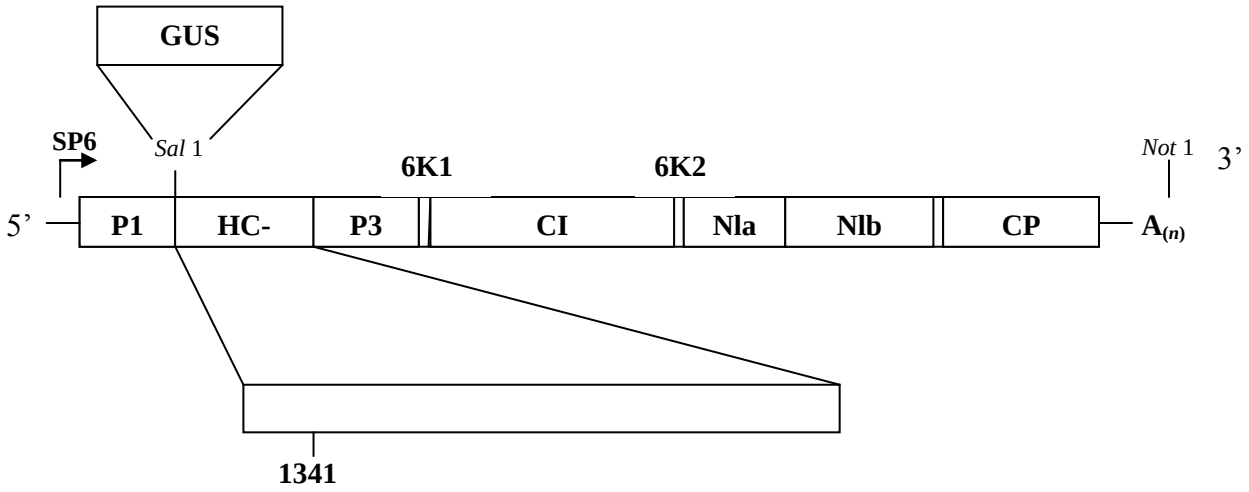
FIGURES

Figure 1



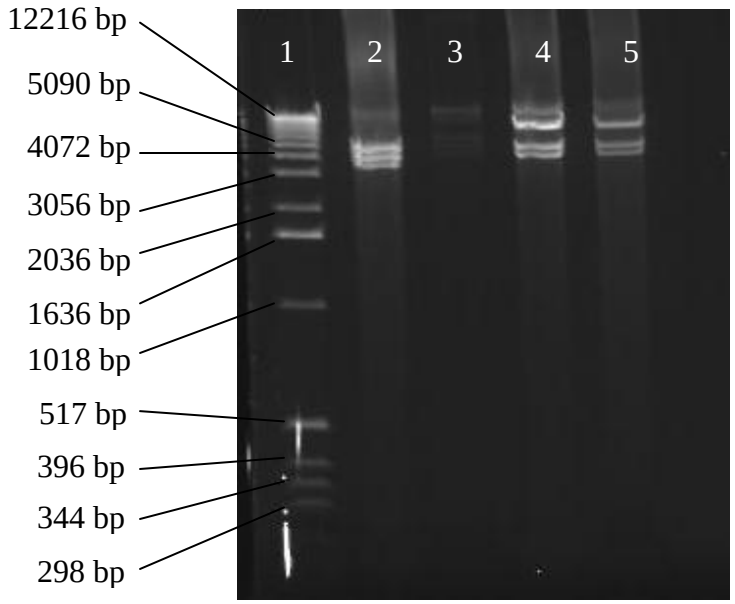
WSMV Sidney 81 and Infectious Clone – The top figure is the genome map of WSMV Sidney 81 the strain used in this experiment. The bottom figure is a diagram of the WSMV infectious clone in the low copy plasmid pACYC-177 (pACYC-WSMV), with a SP6 promoter. The infectious WSMV clone represents the S1RN construct and Gus1RN is the same construct except with a GUS sequence placed between P1 and HC-Pro.

Figure 2



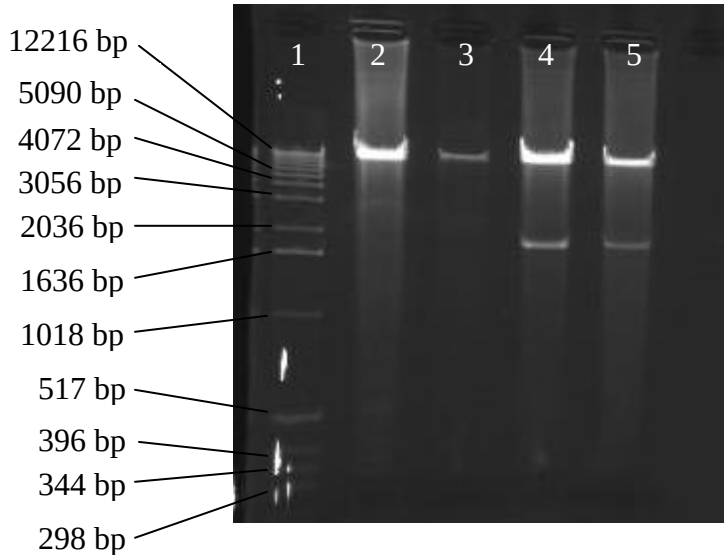
PS81 and Gus46 Genome Constructs – PS81 has a point mutation at nucleotide coordinate 1341 located within the HC-Pro region and no GUS sequence. Gus46 is the above construct with a point mutation at nucleotide coordinate 1341, except a GUS sequence is located between the P1 and HC-Pro protein as shown.

Figure 3



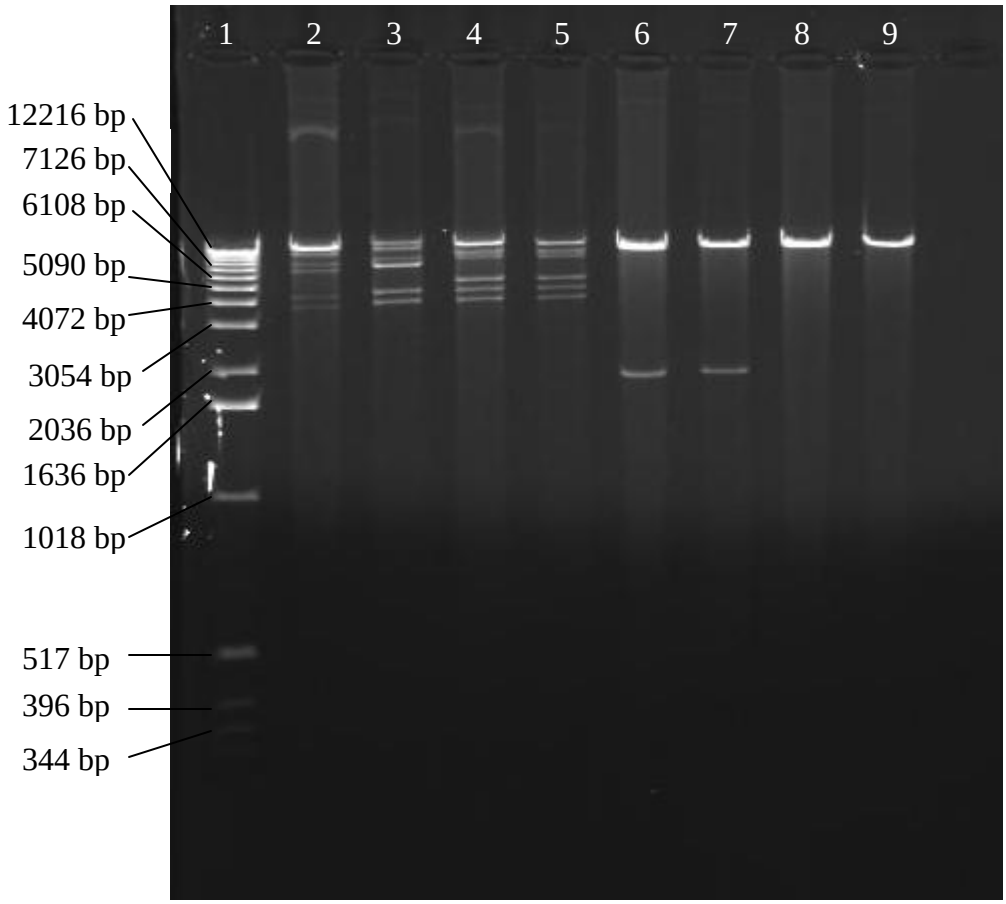
Plasmids Digested with *Sac* 1 - lane 1, 1 Kb ladder, lanes 2-5 are plasmids digested with *Sac* I. Lane 2 is S1RN (1), lane 3 is S1RN (2), lane 4 is Gus1RN (1), and lane 5 is Gus1RN (2) plasmid.

Figure 4



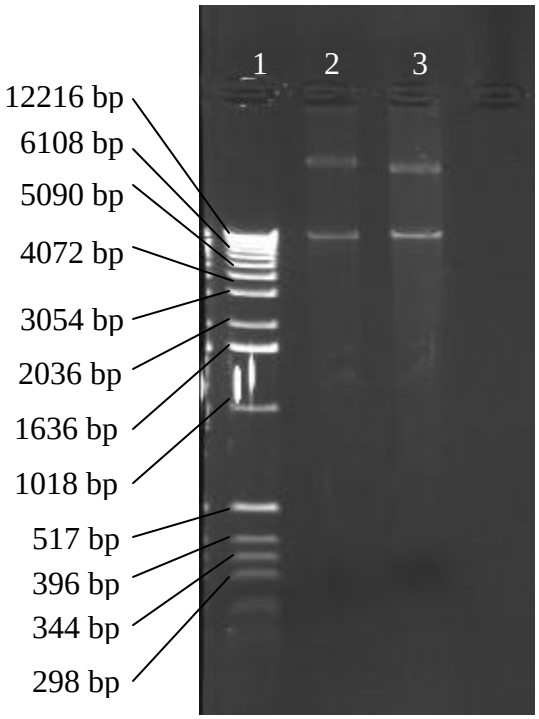
Plasmids Digested with *Sal* 1 – lane 1, 1 Kb ladder, lanes 2-5 are plasmids digested with *Sal* I. Lane 2 is S1RN (1), lane 3 is S1RN (2), lane 4 is Gus1RN (1), and lane 5 is Gus1RN (2) plasmid.

Figure 5



PS81 and Gus46 Plasmids Digested with *Sac* 1 and *Sal* 1– Lane 1 is a 1 Kb ladder, lanes 2 through 5 are plasmid samples digested with *Sac* 1. Lane 2 is Gus46 (3), lane 3 is Gus46 (4), lane 4 is PS81 (3), and lane 5 is PS81 (4). Lanes 6 through 9 are samples digested with *Sal* 1. Lane 6 is Gus46 (3), lane 7 is Gus46 (4), lane 8 is PS81 (3), and lane 9 is PS81 (4).

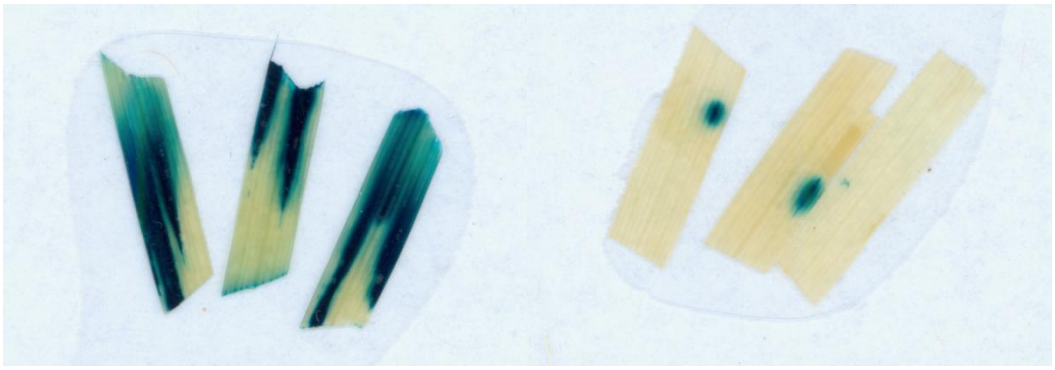
Figure 6



Plasmids Digested with *Not* 1 – lane 1, 1 Kb ladder, lane 2 is Gus46 (2) and lane 3 is PS81 (1).

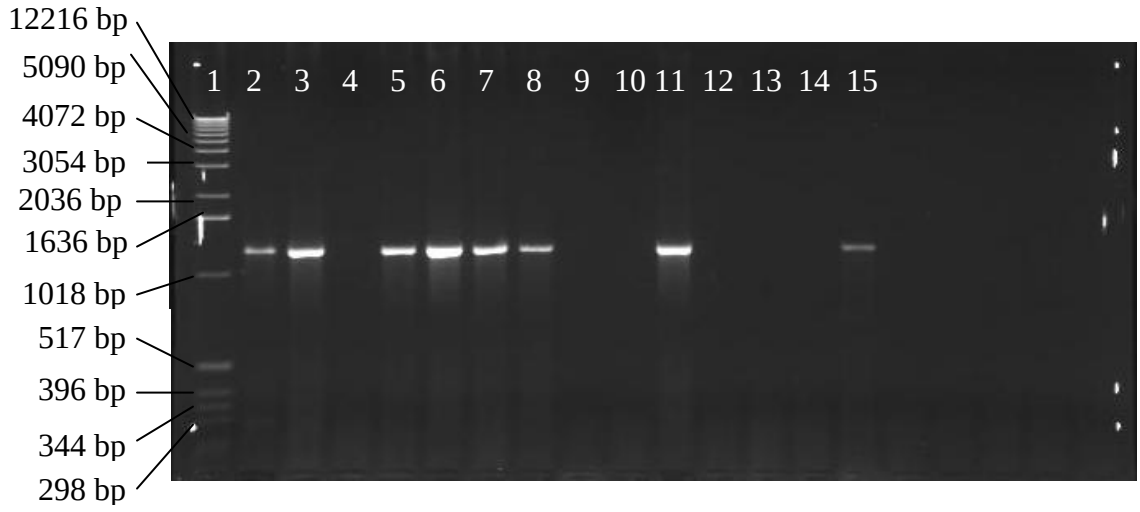
Figure 7

A **B**



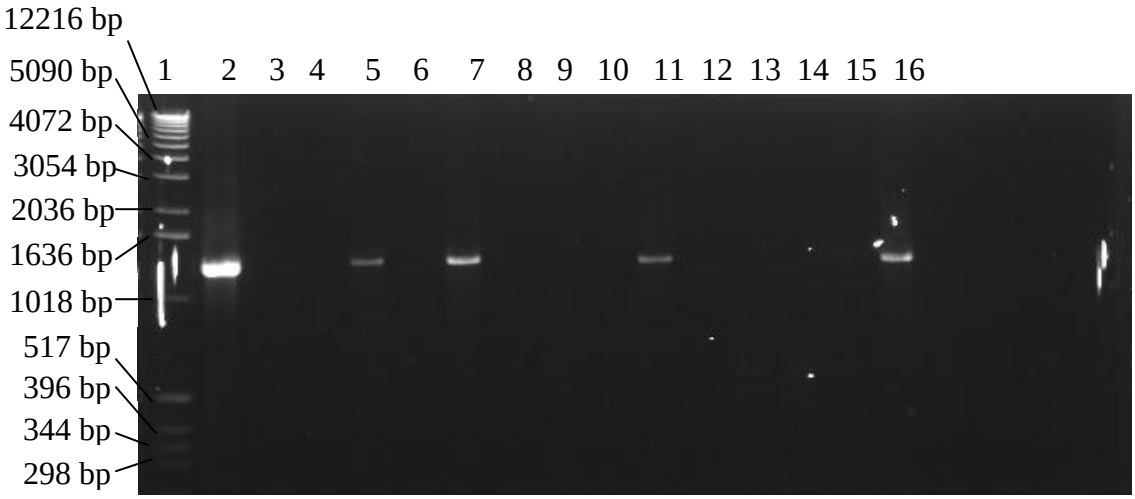
GUS Assay Results – a GUS assay was performed on wheat leaves at 4 dpi
A) Wheat leaves inoculated with Gus1RN. **B)** Wheat leaves inoculated with Gus46.

Figure 8



RT-PCR Products from PS81 Infected Plants – Gel (set 10 #1-18) of *Taq*-PCR products of systemic leaves collected at 20 dpi from wheat plants inoculated with PS81. Lane 1 is 1 Kb ladder, lane 2 is S1RN the positive control and lanes 3, 5, 6, 7, 8, 11 and 15 are positive for PS81 reversion.

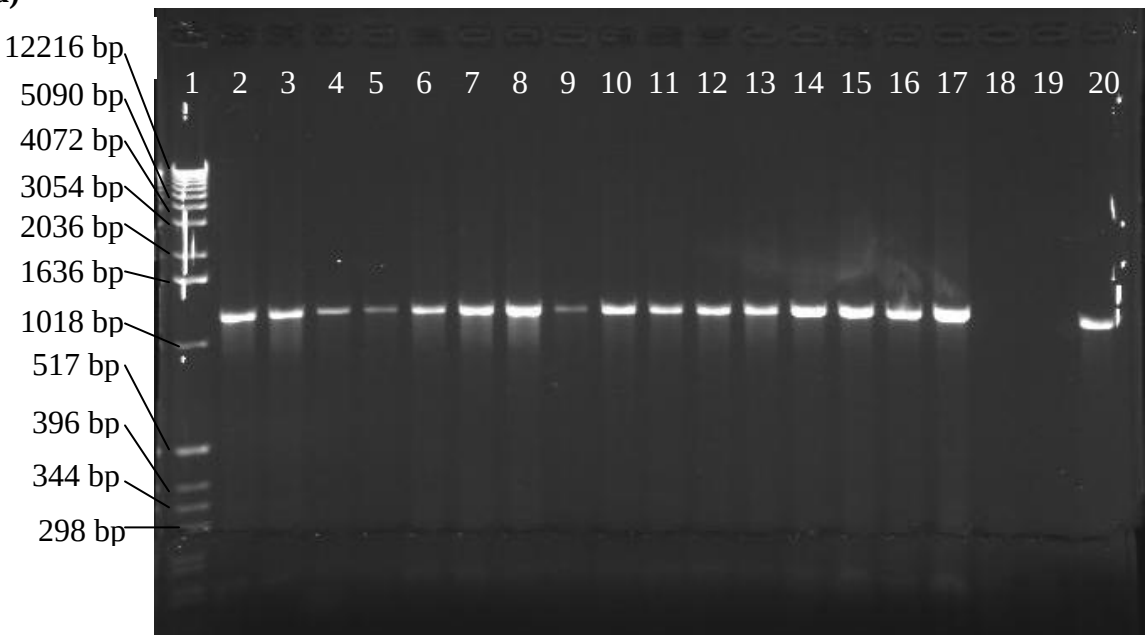
Figure 9



RT-PCR Products from Gus46 Infected Plants – Gel (set 13B #19-36) of *Taq*-PCR products of systemic leaves collected at 28 dpi from wheat plants inoculated with Gus46. Lane 1 is 1 Kb ladder, lane 2 is Gus1RN the positive control and lanes 5, 7, 8, 11 and 16 are positive for Gus46 reversion.

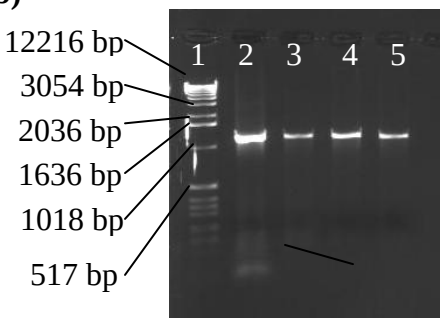
Figure 10

a)



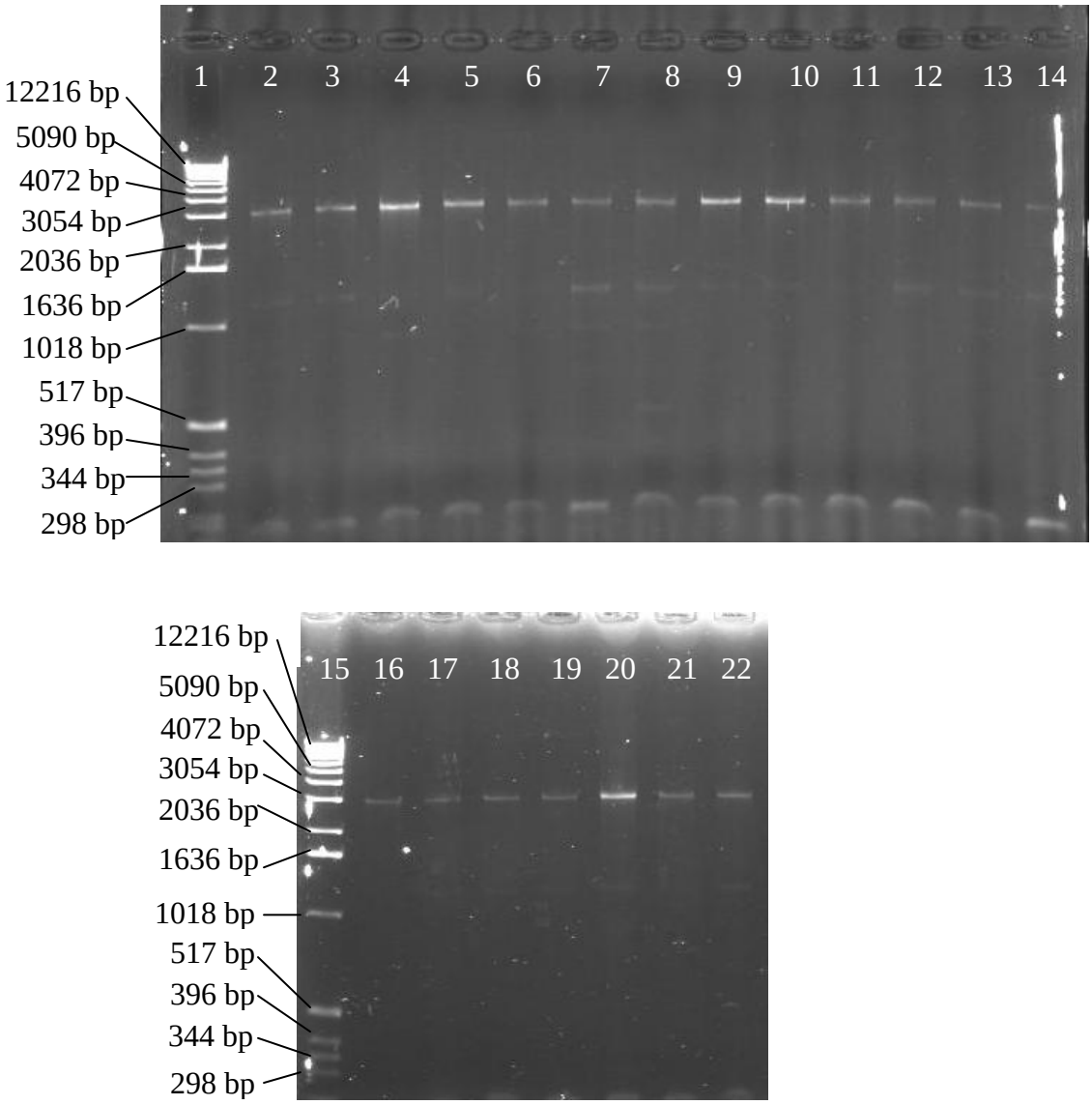
a) RT-PCR Products from Plants Testing Positive for Systemic Infection by PS81 – Gel of 20 positive samples of *Taq*-PCR products that were purified using a PCR Purification Kit to be sequenced. Lane 1 is 1 Kb ladder, lanes 2-20 purified PCR positive PS81 samples. Four samples were taken for each set of PS81. A positive is ~1,200 nt band

b)



b) RT-PCR Products from Plants Testing Positive for Systemic Infection by PS81 – Lane 1 is 1 Kb ladder, lane 2 is S1RN positive control, Lane 3-5 are positive samples missing from Figure 10a

Figure 11



RT-PCR Products from Plants Testing Positive for Systemic Infection by Gus46 – Gel of 20 positive samples of *Taq*-PCR products that were purified using a PCR Purification Kit to be sequenced. Lane 1 and 15 is 1 Kb ladder, lanes 2-14 and 16-22 are purified PCR positive Gus46 samples. A positive is ~3,000 nt band

Figure 12

Tatineni *et al.*, 2010 Real Time PCR readings of WSMV absolute quantification of virus particles

- 14 dpi: 5.55 +/- 4.80 log copies $10^{5.55}$= 354,813 genomes
- 28 dpi: 5.06 +/- 4.66 log copies $10^{5.06}$= 114,815 genomes

Calculation of Genomes per Gram of Plant Tissue

Tatineni Measurement - 4.63×10^{-6} grams of tissue per reaction

14 dpi: $\frac{354{,}813 \text{ genomes}}{4.63 \times 10^{-6} \text{ grams of tissue per reaction}} = 7.66 \times 10^{10}$ genomes per gram of tissue

28 dpi: $\frac{114{,}815 \text{ genomes}}{4.63 \times 10^{-6} \text{ grams of tissue per reaction}} = 2.48 \times 10^{10}$ genomes per gram of tissue

Figure 13

Conversion of Leaf Weight to Leaf Area

- Average Leaf Weight (Table 11): 0.0754 grams per leaf
- Average Leaf Area Infected (Table 3): 451,997 μm^2

$$\frac{0.0754 \text{ grams per leaf}}{451{,}997\ \mu m^2} = 0.000000167 \text{ grams per unit } (\mu m^2) \text{ leaf area}$$

Figure 14

a) Calculation of Grams per Cell

$\frac{0.000000167 \text{ grams per unit } (\mu m^2) \text{ leaf area (Fig. I-13)}}{0.00124 \text{ cells per } \mu m^2} = 0.000135$ grams per cell

b) Calculation of Viral Genomes per Cell

Tatineni's measurements:

14 dpi: 7.66×10^{10} genomes/gram of tissue x 0.000135 grams/cell = 10,341,000 genomes per cell

28 dpi: 2.48×10^{10} genomes/gram of tissue x 0.000135 grams/cell = 3,348,000 genomes per cell

Brakke's measurement:

7×10^{10} genomes/gram of tissue x 0.000135 grams/cell = 9,450,000 genomes per cell

Figure 15

Calculation of Number of Virus Genomes with a Specific Substitution per Cell

- High mutation rate for RNA viruses 10^{-4} per nucleotide per replication
- Expect a 10,000 nt genome

  $10{,}000(10^{-4}) = 1$ mutation per genome

- Chance of a genome with a mutation at a particular site

  1 mutation per genome / 10,000 nt genome = 0.0001

(Number of viral genomes per cell (Figure 14)) (0.0001) =

Tatineni's measurements:

14 dpi: 10,341,000 genomes per cell x 0.0001 = 1,034 (1/3) = 345 copies of the exact mutation within a cell

28 dpi: 3,348,000 genomes per cell x 0.0001 = 335 (1/3) = 112 copies of exact mutation within a cell

Brakke's measurement:

9,450,000 genomes per cell x 0.0001 = 945 (1/3) = 315 copies of the exact mutation within a cell

Figure 16

Equation to Calculate MOI

(reversion per plant) = (# of infected cells per inoculated leaf) x (# of genomes with specific substitution per cell) x $\frac{\text{(\# of genomes moving to adjacent cell)}}{\text{(total genomes per cell)}}$

$$\text{Part 1} = \text{Part 2} * \text{Part 4} * \frac{X}{\text{Part 3}}.$$

- **Part 1** – (reversion per plant) Table 7
  - Gus46 39/184=0.2120

- **Part 2** – (# of infected cells per inoculated leaf) Table 5
  - 566 cells

- **Part 3** – (total genomes per cell) Figure 14
  - 14 dpi: 10,341,000 genomes per cell
  - 28 dpi: 3,348,000 genomes per cell
  - Brakke's: 9,450,000 genomes per cell

- **Part 4** – (number of genomes with specific substitution per cell) Figure 15
  - 14 dpi: 345 genomes
  - 28 dpi: 112 genomes
  - Brakke's: 315 genomes

Figure 17

Calculation of Number of Genomes Moving to Adjacent Cell

Tatineni's measurements:

14 dpi: $0.2120 = 566 * 345 * \frac{X}{10{,}341{,}000}$ = 11.23 genomes move to adjacent cell

28 dpi: $0.2120 = 566 * 112 * \frac{X}{3{,}348{,}000}$ = 11.20 genomes move to adjacent cell

Brakke's measurement:

$0.2120 = 566 * 315 * \frac{X}{9{,}450{,}000}$ = 11.24 genomes move to adjacent cell

Solving for X = number of genomes moving to adjacent cell