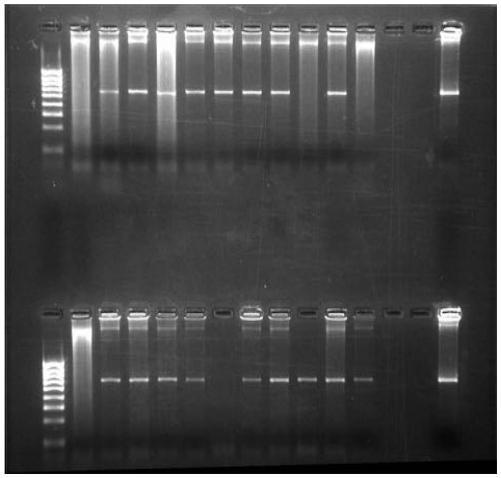
# Wheat virus identification within infected tissue using nanopore sequencing technology



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## ***Background***

- Disease diagnosis is often based on visual assessments; however, abiotic stressors, caused by soil chemical toxicity, nutrient deficiency, heat, and/or drought stress can mimic disease phenotypes.

- Plant viruses are especially difficult to diagnose based on visual symptoms alone.

- In most cases, diagnosis is verified using an ELISA or PCR assay.

## ***Virus Diagnosis Issues***

- Antigen assays can determine the major virus present in a sample but cannot identify specific variants.

- PCR can identify specific SNPs present in the virus population, but PCR is limited to a small region of the genome, nucleotide extraction methods, primer sequences, detection techniques, and PCR results will be skewed towards the dominant virus genotype within the population.

- Sanger sequencing of the virus is time-consuming requiring various techniques to obtain full-length genome sequence.

- Illumina sequencing can produce assemblies of the virus without cloning, but reads are from short fragments, not whole strands.

## ***Nanopore Technology***

- Nanopore technology, such as Pacific Bioscience or Oxford Nanopore, can rapidly sequence full-length DNA, cDNA, and RNA. No cloning is involved.

- Single nucleotide strands remain intact and are rapidly pulled through small pores and sequenced by measuring the change in voltage.

- Oxford Nanopore technology (ONT) was designed for sequencing portability and to be accessible to researchers with limited resources. The ONT device is pocket sized, portable, requires a flow cell for each run, uses simplistic sequencing reaction protocols, and is controlled by a laptop computer (https://nanoporetech.com).

## ***Nanopore Technology continued:***

- In human pathology, ONT was used in the field to identify Ebola, Chikanguanya, and hepatitis C (Greninger et al. 2015). Pathogens were identified very quickly, even with a 24% error rate.

- At 6hrs, MinION produced enough sequence coverage that 90% of the Chickanguanya virus could be assembled at 97-99% accuracy (Greninger et al. 2015).

- In the 2014 Ebola outbreak, ONT produced enough coverage depth to determine an error rate of $1.36 \times 10^{-3}$ (Hoenen et al. 2016).

- *Disclaimer: Mention of trade names or commercial products in this talk is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the U. S. Department of Agriculture*

## ***Wheat viruses***

- Each year the wheat streak mosaic complex (WSM), including *Wheat streak mosaic virus* (WSMV), *Triticum mosaic virus* (TriMV), and *High Plains wheat mosaic virus* (HPWoMV), is responsible for an average of 5 % loss in production (Burrows et al. 2008).

- Wheat plants are often infected with multiple viruses, including *Barley yellow dwarf virus* (BYDV).

- No commercial ELISA detection kits are available for TriMV and other kits need high virus titer for detection.

# Two Potyviruses infecting wheat

*Wheat streak mosaic virus*

(Birmingham Virology, UK)

*Triticum mosaic virus*

(Dallas Seifers)

## ***Possible new WSMV strain***

- In 2015, 'Clara CL', a *Wsm2* derivative of hard winter wheat RonL, was found to have severe mosaic symptoms and stunting throughout a field in Hamilton County, KS. ELISA suggested only WSMV was present in plant tissue.

- In 2017, three more sites, in western Kansas, planted with *Wsm2*-containing lines, were identified with WSM-like symptoms and ELISA verified to have WSMV.

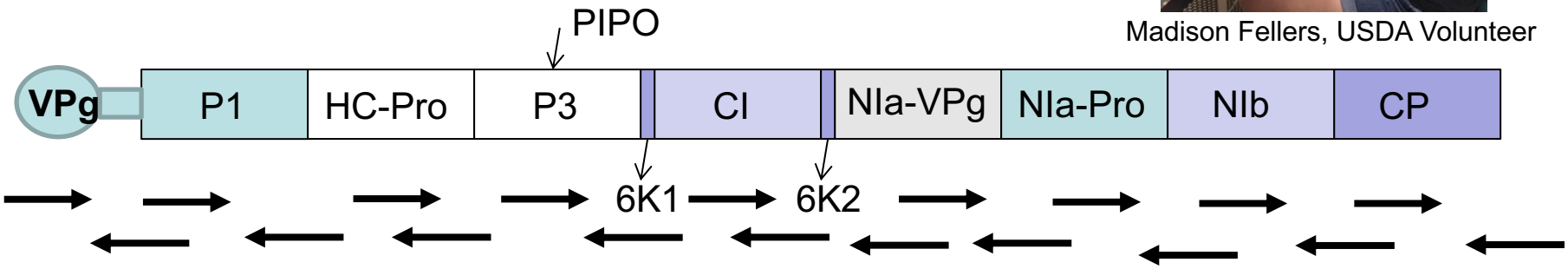
  Greely County, Variety: Oakley CL
  Wichita County, Variety: Joe
  Wallace County, Variety: Oakley CL

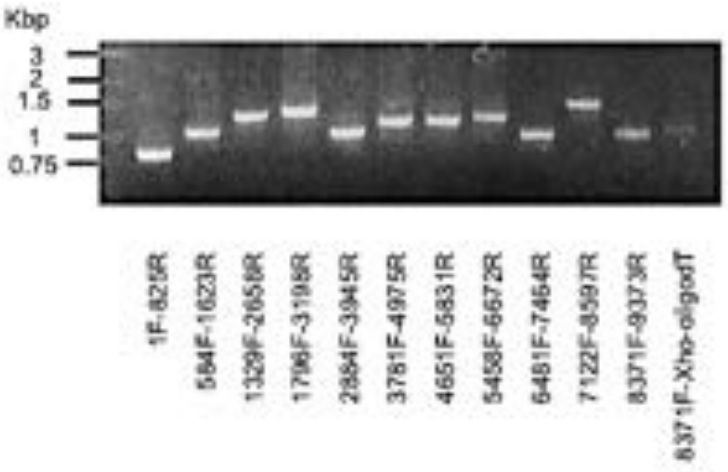
- ***A confident diagnosis could not be made concerning the possibility of a mixed infection or the emergence of a new virus.***

# *Cloning and Sequencing WSMV*

- The 2015 Clara CL WSMV sample was initially sequenced using a classical approach of PCR, sub-cloning.

Madison Fellers, USDA Volunteer




- Two different WSMV virus assemblies were found.

- WSMV_Hm_1
  9,384 nt, 98.6% identical to WSMV-S81.

- WSMV_Hm_2
  9,381 nt, 98.5% identical to WSMV-S81.
  3 nt deletion at position 8311.

Fellers et al., 2019, Plant Dis 103:2199

## ***Approach***

- Total RNA was isolated using *mirVana*® RNA isolation kit from symptomatic wheat tissue taken from fields Hamilton Co., Greely Co., Wallace Co., and Wichita Co. Kansas, and from infected tissue containing lab strain WSMV-MHK.

- All samples were ONT sequenced by creating full length cDNA with both random 6-mer and oligo-d(T) 1$^{st}$ strand primers

- 2$^{nd}$ strand synthesis with NEBNext enzyme mix; PCR amplified using ONT PCA adapters and LongAmp® *taq* enzyme mix; sequencing library was prepared according to the ONT 1D-cDNA sequencing kit; samples were loaded onto a MinION 107 v9.5 Flow Cell.

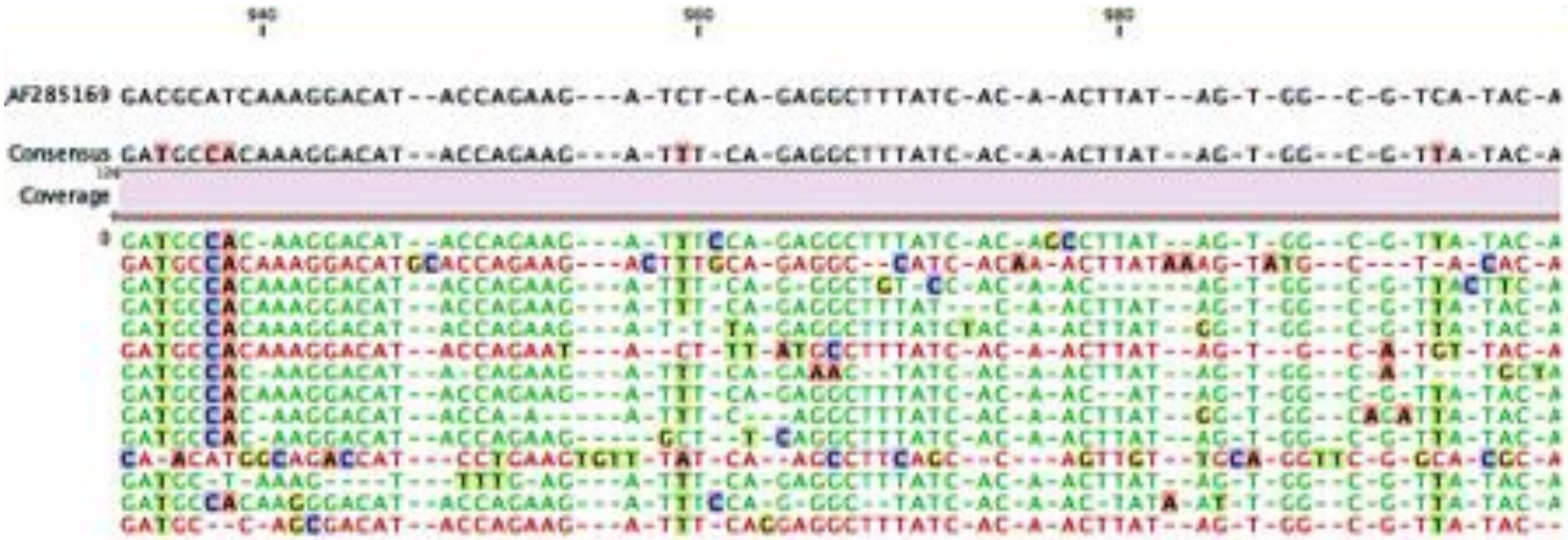
- Sequence was base called using *albacore* v1.7.3, trimmed with *porechop* v0.2.3, assembled and BLAST analyzed with CLC Genomics workbench v11.0.

Table 1. cDNA sequencing results from Oxford Nanopore Technology MinION® flow cells of virus infected wheat samples. Adapters were trimmed and the reads were aligned to a set of 25 cereal viruses including HPMoV.

| | | | | | | Avg fold coverage* | | |
|---|---|---|---|---|---|---|---|---|
| Location, Year | Variety | Run time | Total reads | Total bp | Avg. length | WSMV | TriMV | BYDV |
| Hamilton Co., 2015 | Clara CL | 24 hr | 2.1 million | 2.4 Gb | 1,158 bp | 1,284X | 600X | - |
| Greely Co., 2017 | Oakley CL | 12 hr | 1.1 million | 1.1 Gb | 1,005 bp | 896X | 162X | 247X |
| Wichita Co., 2017 | Joe | 6 hr | 760,226 | 0.810 Gb | 1,066 bp | 374X | 101X | 17.7X |
| Wallace Co., 2017 | Oakley CL | 2 hr | 157,798 | 0.113 Gb | 721 bp | 18.1X | 4.3X | 8.6X |
| WSMV-MHK | Karl 92 | 4 hr | 173,813 | 0.323 Gb | 1,863 bp | 45.3X | - | - |

* Average fold coverage is based on the average number of times specific virus nucleotides are sequenced across the virus genome.

# ***Strand alignments showing diversity***



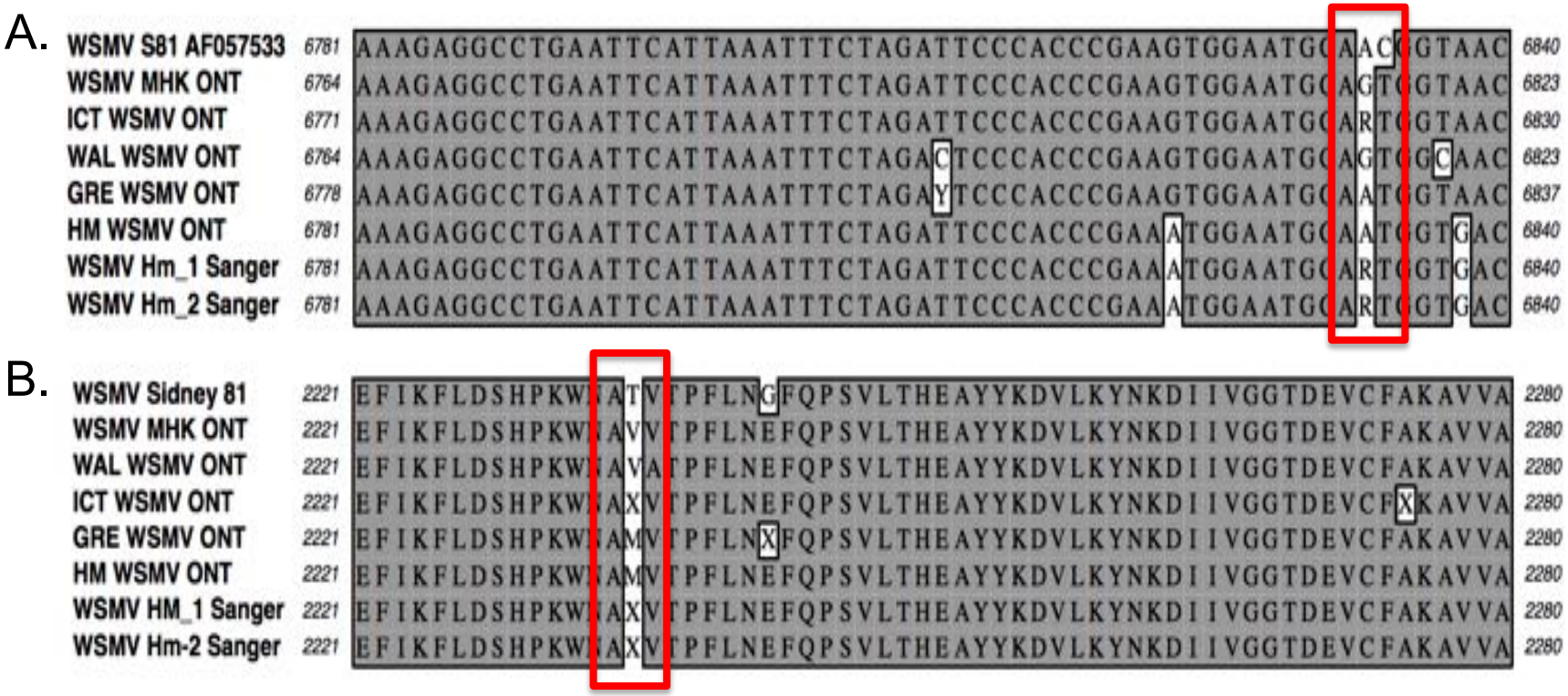

ONT technology allows for the identification of individual genomes and could be used to study population variance within a plant and field.

Table 2. Nucleotide identity (%) of virus isolates from sampled wheat tissues. Viruses were assembled and aligned using Clustal W to standard isolates. Columns represent isolates of *Wheat streak mosaic virus* (WSMV), *Triticum mosaic virus* (TriMV) and *Barley yellow dwarf virus* (BYDV), respectively.

| | **Virus isolate used for comparison** | | |
|---|---|---|---|
| Tissue source | WSMV-Sidney 81* | TriMV U06-123** | BYDV-PAV*** |
| Hamilton Co. | 99.6 | 92.1 | - |
| Greely Co. | 99.6 | 92.2 | 95.0 |
| Wichita Co. | 99.5 | 92.2 | 79.4 |
| Wallace Co. | 99.4 | - | 93.7 |
| WSMV-MHK | 99.5 | - | - |

* AF057533, Stenger et al., 1998;
** FJ263671, Fellers et al., 2009
*** EF043235, Beckett and Miller, 2009, Unpublished

# ***Virus Sequence Allignments***




ClustalW alignments of A) nucleotides and B) amino acids of assembled WSMV variants found in infected wheat tissue against WSMV Sidney 81 (AF057533). Red boxes highlight suspect changes in the viruses that may be the cause of virulence shifts in WSMV resistant varieties.

## ***Conclusions:***

- In the plant diagnostic lab results with ELISA confirmed WSMV, was inconclusive for BYDV and had a single positive reaction for the presence of HPWoMV.

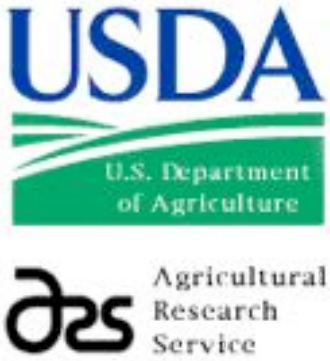
- A six week project of PCR, cloning, and Sanger sequencing confirmed the presence of two genotypes of WSMV.

- For ONT, from sample to sequence took 1.5 days, and a 2 h run could provide enough sequence to accurately identify viruses, but required at least 40X coverage to assemble a genome.

| | | |
|---|---|---|
| - Cost: | Minion device | $1000 |
| | Flow Cell | $900 |
| | Sequencing Rxn | $100 |
| | Small PCR machine | $2500 |
| | Laptop | ~$1500 |
| | (New Flongle Cell $90) | |

## My Collaborators

Madison Fellers, BS, CTRS
Dr. Erick De Wolf
Christian Webb, MS
Dr. Jessica Rupp
Zane Kohl, HS Honor Student

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