National Clean Plant Network – Sweetpotato

Virus Workshop, Greenbelt, MD

September 16, 2015

Attending: Jorge Abad (USDA, PGOP), Chris Clark (LSU), Emily Ringelman (LSU), Zvezdana Pesic van Esbroeck (NCSU), Christie Almeyda (NCSU), Kai-shu Ling (USDA, Charleston), Yan Meng (Alcorn), Sue Sim (FPS, UC Davis), Mike Melzer (UH), Meher Al Rwahnih (FPS, UCD), Bob Jarret (USDA, Griffin), Sathish Ponniah (UAPB), Heather Brown (USDA, PGQP), Jennifer McCallister (USDA, PGQP), Prat Bandla (USDA, PGQP), Maria Soto-Aguilar (Danforth), Ruhui Li (USDA, ARS), Jeff Main (MSU)

Jorge Abad welcomed all to the meeting on behalf of USDA, APHIS and stated the goal of the meeting to develop a list of viruses affecting Sweetpotato, know about geographic distribution and transmission, and how best to detect those viruses.

Maher Al Rwahnih – made a presentation based on his experience with grape viruses. He indicated that there are 6-8 viruses defined as 'regulated' even though many more are tested for. Biological tests can be high cost, requiring considerable greenhouse inputs, and is limited by the availability of hosts that express symptoms reliably to any given virus.

Next Generation Sequencing (NGS) is especially useful for detecting unknown etiology diseases and/or unrecognized viruses and for virome analysis.

Biological indexing failed to detect 5 out of 15 regulated viruses in certain hosts (that were detected by NGS and confirmed by qRT-PCR.

Conventional virus testing cost ~$1,900 and took 2-3 years. NGS cost ~$900 (that price is still going down) and required a few months.

There are technical challenges for using NGS –

- Need to standardize techniques, what type of extract to use, standardize approach to bioinformatics (main companies = CLCbio or DNAStar).
- Has found a DNA virus (GRBaV) with NGS, but did not use any enzymatic digestion.
- 7-12% unidentified reads – do they include unknown viruses? – Wu et al paper to identify potential virus sequences independent of homology to known.

Canuti and van der Hoek – are we genome collectors? What is the biological significance of sequences found? To verify biological significance one should ideally:

1. Determine graft transmissibility

2. Fulfill Koch's Postulates, if possible
3. Determine spread and distribution of the sequence
4. Assess agronomic significance (symptoms)

Grapevine red blotch associated virus is an example of a virus first found with NGS but confirmed biologically. Also found in a herbarium sample from 1940.

FPS will run biological tests side-by-side with NGS for grape for several years before deciding whether to discontinue biological testing. At FPS test for 37 viruses, essentially any that they can get positive standard material for.

Ruhui Li made a presentation on her recent research to improve detection of sweetpotato viruses.

She is using GWB, a Beauregard collected in North Carolina as a 'golden plant' infected with six viruses from NC, including all four potyviruses (SPFMV, SPVG, SPVC, SPV2), *Sweet potato chlorotic stunt virus* (WA=US strain), and *Sweet potato leaf curl virus*.

She is using HiSeq RNA and mapping to siRNA - 244 sequences: All_SP_Virus_Ref_Seq

- These methods can detect all 6 viruses
- Her results indicate there may be multiple sequences of SPFMV.
- Detected one poty by siRNA sequencing that could not be amplified by PCR suggesting a possible false positive by siRNA.
- Used a subtraction analysis approach to avoid dependency on known sequence homology. Removed healthy host reads, removed known virus reads, de novo assembly.

siRNA attributes:

- Higher % of reads
- Slightly lower price
- Small contigs
- False positive
- Missing viroids
- More lab verification work

Hiseq attributes:

- Large contigs
- Identification of variants

- Differentiation analysis
- Lower reads for DNA viruses

Conclusions: both are sensitive, rapid, and reliable for detection of viruses. Hiseq sequencing is useful in identification of novel pathogens/viruses.

Got graft transmission to *Ipomoea setosa* of something that caused chlorotic spots on setosa.

Kai-shu Ling presented some of his previous research with the sweetpotato begomoviruses and ongoing research with NGS in sweetpotato.

47 of 701 accessions in the USDA sweet potato germplasm repository were positive for SPLCV. Many of these have not yet been cleaned up.

The qPCR primer/probe sequence developed by Kokkinos and Clark, while useful for quantification of the isolate for which it was developed, is not conserved, but the new Ling et al. primer/probe is conserved among 10-12 isolates and therefore a better system for detection purposes.

sRNA deep sequencing has identified sequences in sweetpotato collected at Charleston with homology to 22 begomoviruses, 4 potyviruses, 3 badnaviruses, and 1 mastrevirus. It also found a hit for Sweet potato vein mosaic virus (64% coverage).

He has determined sequences using NGS for 14 putative isolates of SPFMV and SPVC collect and biologically characterized in Louisiana by Chris Clark. These are in the initial stages of confirmation by a graduate student at LSU, Favio Herrera.

He also discussed potential priorities for using NCG for NCPN-SP.

Chris Clark presented a summary of past work on the viruses known to occur in sweetpotato in the U.S. as well as viruses known to occur elsewhere.

'Naturally infected' plants have yield reductions ranging 25 to 40%. Four potyviruses: *Sweet potato feathery mottle virus* (SPFMV), *Sweet potato virus G* (SPVG), *Sweet potato virus C* (SPVC), and *Sweet potato virus 2* (SPV2) are common in sweetpotatoes in the U.S. While several strains are known within SPFMV, there is little information on which occur in the U.S. and which can cause russet crack on contemporary cultivars. Data has been obtained on effects of SPFMV, SPVG, and SPV2 and individually, they were found to have only slight effects on yield of sweetpotato, but as multiple infections accumulate in plants, yield reductions of about 15-20% have been documented. It appears that some other entity contributes to yield losses, but it is not yet known whether that is SPVC or an unknown virus or other pathogen.

Sweet potato leaf curl virus (SPLCV) was initially found only in purple ornamental sweetpotato cultivars and some old heirlooms in breeders' collections but it can reduce yield of Beauregard sweetpotatoes about 30% without causing discernible foliar symptoms. Subsequently, two other

species of begomovirus have been reported in the literature: *Sweet potato leaf curl Georgia virus* and *Sweet potato leaf curl South Carolina virus* but little is known of their biological properties.

Sweet potato chlorotic stunt virus (SPCSV) was identified in a single accession in the germplasm repository and in isolated field samples from North Carolina on two occasions. However, historical records suggest the possibility that the 'Georgia Mosaic' that was presumably eradicated in the early 1960s and the 'Sweetpotato Mosaic' that occurred in the 1920s-30s may have involved SPCSV but occurred before technology existed to characterize the virus using currently accepted principles. When American cultivars were grown in East Africa where SPVD (the syngergistic interaction of SPCSV and SPFMV) is prevalent, 80-90% yield reductions were measured.

Many other viruses occur around the world that have not been reported in the U.S.

Zvezdana Pesic van Esbroeck and Christie Almeyda gave a presentation on sweetpotato virus indexing in North Carolina.

Testing consists of grafting to *Ipomoea setosa* and testing for:

- the four common potyviruses (SPFMV, SPVG, SPVC, and SPV2) using the PCR protocol of Li et al., 2012
- Geminiviruses using the PCR protocol of Li et al., 2004
- The West African strains of SPCSV using a RT multiplex quantitative PCR developed by USDA, APHIS.

Testing is done at four stages: 1) breeders material before therapy, 2) testing of tissue culture plants after meristemming, 3) testing after greenhouse establishment, and 4) testing after grafting to *I. setosa*.

They also have used a multiplex PCR test for begomoviruses, solendoviruses, and cavemoviruses.

When doing meristemming, they aim for ≤0.2 mm and for that reason use microsurgical scalpels to cut the meristems

Maher al Rwahnih gave a brief overview of the approach used with grapes to develop their list of regulated viruses:

- Phantom diseases or viruses – viruses with no sequence information, strains or positive standards with which to work.
- 'Real viruses'
    - Viruses with a demonstrable effect on the plant
    - Viruses with recommended detection method

The group then discussed what viruses should be considered for targeting for sweetpotato and what detection methods were most effective for sensitive detection of these viruses. There was also some discussion of knowledge gaps that affect testing reliability. These discussions led very quickly to identification of six viruses as those that should be targeted in the U.S. and a table was generated listing those viruses, the preferred detection method and alternative acceptable detection methods. The group felt that more information is needed on diversity of SPFMV in the U.S. The group also felt that although more than 10 species are recognized by ICTV and 22 distinct viruses recognized in recent NGS studies that the geminiviruses should be considered as a group for now. However, there remains a need to verify that the PCR and multiplex qPCR protocols will reliably detect each of the strains of geminivirus present in the U.S.

Virus	Detection	Alternative Det.	Knowledge gaps
SPFMV	Generic PCR Ha et al. 2008	Li et al. 2012 Multiplex PCR	More information is needed on diversity.
SPVG	Generic PCR	Li et al. 2012 Multiplex PCR	
SPVC	Generic PCR	Li et al. 2012 Multiplex PCR	
SPV2	Generic PCR	Li et al. 2012 Multiplex PCR	
SPCSV	CPHST qPCR		
Geminiviruses	Li et al. 2004 PCR	Ling et al qPCR	Need to verify against multiple species/strains

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September 17, 2015

Reconvened at BARC Building 580

Joe Foster welcomed the group to PGQP and gave an overview of the various operations and functions of the lab.

Jorge Abad related the functions of his group's sweetpotato quarantine activities to the overall activities of PGQP.

Joe Foster oversees postentry quarantine and all the other federal quarantine programs and has sole power to release accessions from quarantine.

Jorge recommended checking the e-CFR Electronic Code of Federal Regulations (7 CFR 319.37-2) to see the specifics of quarantine regulations.

Sweetpotato botanical seed is not yet regulated, but in light of Korean paper on seed transmission of SPLCV, it may soon be. Materials are imported only via processing in PGQP or Controlled

Import Permit (can allow early release under controlled conditions for specific testing if originating from an entity recognized as a source of properly tested materials, e.g. CIP for sweetpotato). NAPPRA – conditions allowing import pending pest risk analysis.

The Quarantine Process:

- Accept requests and assign slots for imports
- Coordinate importation
- Shipment arrival at the PIS
- Shipment release from PIS to PGQP
- Treatment and establishment of clones

Sweetpotato accessions received – a shoot tip is established in tissue culture and the base of the plant is used for testing. Currently, about 90% of sweetpotatoes are received as tissue cultures. Accessions are all released as tissue cultures.

Jorge made several comments about sweetpotato operations at PGQP:

- Prat Bandla has been trained for doing cryotherapy but the procedure has not yet been adopted at PGQP.
- Crindi Loschinkohl has refined methods for grafting to I. setosa, she puts water on scion, uses VetWrap and covers with plastic bag for a couple of days after grafting.
- They grow positive controls at exactly same time and conditions as test plants.
- They compared qPCR with TIB-8 for three years for testing for SPCSV before adopting the qPCR method.

Several observations were raised during discussion:

- CIP is replacing biological tests with Deep Sequencing.
- The requirements EPPO instituted for testing for PSTVd can be resolved. Research by Jorge and Kai-shu indicates PSTVd does not systemically infect sweetpotato. Furthermore, it is considered eradicated in the US.
- Iso thermal amplification may be ideal for eliminating positives at pretesting and/or for field testing.
- Digital PCR is very sensitive.
- To account for sequence variability in some grape viruses, multiple probes for qPCR are used.

Group Discussion

The discussion focused on how the group felt we should proceed with testing for the six target viruses. The following actions/approaches were agreed to:

- Jorge will provide CPHST work instructions for the qPCR detection for both WA and EA strains of SPCSV and get clearance for its use and for properly crediting the developers.
- Everyone will use the same positive standards. The 'GWB' plant infected with all six target viruses will be used. Everyone will need a 526 permit for curation for each virus to obtain this material.
- Ruhui will provide protocol for saving dry pellet extracts of SPCSV standards.
- Kai will prepare work instructions for qPCR for swepoviruses
- Ruhui will prepare work instructions for PCR for geminiviruses, Cl-Rev for potyviruses and potyvirus multiplex.
- Mike will prepare work instructions for Nib 2-F/3R PCR for potyviruses.
- Ruhui will provide either Nancy Hall or GWB as a positive control for the four potys, SPCSV, and SPLCV.
- Maintaining the crude lysate from extractions in -80 is a means of backing up standards.
- Testing after grafting to *I. setosa* (4^{th} virus testing on Christie's chart), will be considered a mandatory test, but additional testing is optional.
- NGS testing should be done on sweetpotato.
- Sampling strategy –
    - Leaves of different stages (top, middle, bottom of plant) – tissue from base of leaf
    - Roots should also be sampled since some potyviruses appear to replicate to a greater extent in roots than leaves.
- Extraction- either Qiagen or Ruhui's CTAB procedures are acceptable.

Other Discussion:

All files/documents will be provided initially both by email and by uploading to the SharePoint site. After that, documents will be available on SharePoint. A refreshed on how to access SharePoint will be sent and email invitations will be sent to all meeting participants who are not currently on the site. Reminder: invitations expire after 3-4 days, so when you receive the invitation, please sign on to the site within that time. After the first access, subsequent access is relatively easy.

The group felt we need a baseline to be certain what viruses occur in the US, to help learn the diversity of virus sequences of target virus groups (especially potyviruses and geminiviruses), and to verify the apparent absence of other viruses of concern known to occur in other countries on sweetpotato. To this end, the group concurred that:

- We need to find old sources predating clean seed programs
    - Old heirloom cultivars and breeding lines
    - Perennial morning glories from sweetpotato production areas
- Samples could be bulked for NGS
    - Start collecting samples now
- Kai-shu will provide NA extraction protocols
    - Extract, save aliquot, send aliquots to Kai and Ruhui, may need to do validation
- Kai-shu is interested in NGS, Ruhui in Hi-Seq and siRNA analyses.
    - Funding will be required to do adequately.
        - Explore all avenues for funding, Chris will consult NCPN admin team about possibilities.

We extended our thanks to Jorge Abad and the PGQP group for hosting a very productive meeting. Adjourned at 4:15 PM