Development of CRISPR-based next-generation diagnostic method to evaluate *Beet necrotic yellow vein virus* causing rhizomania in sugarbeet

Vanitha Ramachandran*, John Weiland, and Melvin Bolton
USDA-ARS, Sugarbeet & Potato Research Unit, Edward T.Schafer Agricultural Research Center, Fargo, ND

Rhizomania is an important disease of sugar beet caused by *Beet necrotic yellow vein virus* (BNYVV) affects sugarbeet production and growers' economy. The disease is a major concern because of the emergence of resistance-breaking (RB) strains of BNYVV in the Red River Valley and southern Minnesota sugar beet growing areas and around the world within the last 15 years. Rhizomania disease management measures principally rely on resistance genes bred into commercial varieties specifically developed against BNYVV (Rush et al., 2006). Accurate and sensitive detection of BNYVV in plants and infected fields soils are crucial in appropriating management strategies that include varietal selection, non-host crop rotations, and evaluating resistance levels of sugarbeet breeding lines. Firstly, it can identify infected soil to adopt disease management strategies. Secondly, it can distinguish the truly resistant sugarbeet breeding lines from those of partially resistant lines. Enzyme-linked immunosorbent assay (ELISA), a protein-based detection technology has been used for many years for field soil evaluations because of the ease of implementation and availability of reagents commercially (Torrance et al., 1988).

In this study, we developed a new molecular diagnostic method based on CRISPR-Cas12a system termed DETECTR (DNA Endonuclease Targeted CRISPR Trans Reporter) technology (Chen et al., 2018) for detecting BNYVV in the roots of sugarbeet. Template DNA amplification of viral fragments under isothermal conditions is crucial for developing CRISPR-Cas12a based diagnostics method. We have developed an inexpensive isothermal one-step reverse-transcription (RT) recombinase polymerase amplification (RPA) method and confirmed the sequence identity of the RT-RPA amplicon representing the BNYVV sequence. Further, the CRISPR-based BNYVV detection method was evaluated, and the sensitivity determined in the roots of sugarbeet baited for rhizomania using field soils.

Materials and Methods

Soil samples were obtained from the sugarbeet production areas of North Dakota and Minnesota courtesy of agriculturists from the Southern Minnesota Beet Sugar Cooperative (Renville, MN). Sugarbeet seeds, susceptible variety used obtained from SESVanderhave (Fargo). For healthy control, susceptible sugarbeet seeds were planted into Sunshine Mix with sand of 1:1 ratio (Sungro Horticulture, MA). Slow-release fertilizer (Sungro Horticulture, MA) was added following the manufacturer's instructions. Plants were grown in a greenhouse under standardized conditions at 24°C/18°C day/night with 8 hours of supplemental light per day, and water was added directly as needed. Six weeks after planting in infested soil, plants were harvested, and a root sample consisting of 2-3 plants was taken from each pot. Roots were washed gently in a tray containing water taking care to retain fine root hairs, damp dried on paper towel, and stored at -80°C until used for RNA extraction.

One hundred mg of cleaned root tissue was ground using a pulverizer (SPEX, Fisher Scientific, MA), and then total RNA was extracted using RNeasy Plant mini kit (Qiagen, MD), with final RNA concentration determined using a Nanodrop (Thermo Fisher Scientific, MA). Equal concentration of total RNA from healthy and rhizomania infected roots were used for setting up RT-RPA reactions. Reverse transcription-recombinase polymerase amplification (RT-RPA) reactions were performed using TwistAmp liquid basic kit (TwistDx, Cambridge, UK). Primers were designed and synthesized from Integrated DNA Technologies (IDT, IA) for amplifying a 465 bp fragment of BNYVV RNA-1 (Weiland et al., 2020). RT-RPA reactions were setup using total RNA (100 ng) from rhizomania baited roots and healthy roots separately in a 50 µL reaction containing forward (VR-1) and reverse (VR-2) primers (Table. 1), each 2.5 µL (10 µM), 2X reaction buffer 25 µL, dNTPs 4 µL (10 mM), 10X basic E-mix 5 µL, 20X core reaction mix 2.5 µL (TwistDx, Cambridge), and 2 µL of M-MuLV Reverse Transcriptase (NEB, MA), and MgOAc 2.5 µL and remaining volume adjusted with nuclease-free water. After gently mixing and collecting the contents of the tubes with a brief spin, the reactions were incubated at 42°C for 60 minutes. To visualize the RT-RPA products, an aliquot of the RPA reaction was transferred to a new tube and to that EDTA was added to a final concentration of 20 mM and held at room temperature for 5 minutes. After a brief spin the contents were loaded onto the agarose gel containing SyberSafe stain (Invitrogen, MA). Following electrophoresis, DNA products were visualized using Chemdoc (Bio-Rad, CA). Gel-elution of the RT-RPA product was carried out using a Gel-extraction kit (Qiagen, MD) and subjected to Sanger sequencing (MCLAB, CA).

For CRISPR-based reporter assay, ribonucleoprotein complex was pre-assembled using Cas12a (30 nM), guide RNA (40 nM), and ssDNA reporter with fluorophores 5' 6-Carboxyfluorescein and 3' Black Hole Quencher-1 (30 nM) in 1X concentration of Cas12a cognate buffer at 25°C for 10 min. For BNYVV detection from sugarbeet root samples, first RT-RPA was conducted using 100 ng of total RNA extracted from sugarbeet root tissue in a volume of 50 µL using the primers VR-1 and VR-2. The reactions were incubated at 37°C for 60 min and output fluorescence signal was measured at 485 nm excitation and 535 nm emission in a Tecan Spark Ultra plate reader (TECAN, Switzerland). The RT-RPA reactions were diluted up to 10^{-5}, and from each dilution 5 µL was used for the CRISPR-assay. For CRISPR assay background noise alleviation the no template control (NTC) reaction was included in the assay, which was subtracted from test samples prior to plotting the graphs.

Results and Discussion

Rhizomania disease baiting in sugarbeet roots was accomplished by growing plants using rhizomania infested soil. The presence of rhizomania in the soil investigated in this study was confirmed by ELISA testing for BNYVV presence in a soil-baiting assay. Under greenhouse conditions, sugarbeet grown in pot containing soil obtained from field showed yellowish foliar phenotype, while healthy plants grown in potting mix (no field soil) the leaves remained noticeably more-green (Fig. 1A). The representative root phenotypes of rhizomania infected verses healthy sugarbeet plantlets are shown in Figure 1B. Healthy plants' root is large with dense rootlets, whereas rhizomania diseased root is thinner with less-dense rootlets (Fig. 1B).

We developed RT-RPA-based isothermal amplification of BNYVV targeting RNA-1. To accomplish RT-RPA of BNYVV, we designed primers that amplified a 465 bp fragment of BNYVV RNA-1 in a single tube using reagents

from TwistAmp liquid basic kit along with M-MuLV reverse transcriptase from total RNA extracted from sugarbeet root samples. The RT-RPA products were visualized on the gel after treating it with 20 mM EDTA to a final concentration upon loading even 5 and 10 µL out of total 50 µL reaction volume (Fig. 1C and D). The RT-RPA analysis of total RNA isolated from sugarbeet roots baited for rhizomania from infected field soil revealed the production of an amplification product of 456 bp fragment as expected (Fig. 1C and D). No such amplification product was obtained in the root samples from healthy sugarbeet controls. Absence of amplified product with no template control (NTC) revealed the specificity of reagents used in the RPA assays. Next, to confirm the sequence authenticity, the RT-RPA amplified fragment was gel-purified, and Sanger sequencing analysis revealed the amplicon indeed carrying BNYVV RNA-1 sequence as expected. Taken together, we have developed an isothermal one-step RT-RPA assay to amplify BNYVV from sugarbeet roots baited for rhizomania from field soil, optimized conditions to visualize the RT-RPA products on gel and confirmed the identity of underlying sequence useful for downstream molecular analysis.

Next, we investigated application of the developed CRISPR-Cas12a based virus diagnostic assay for detecting BNYVV in the roots, the most highly impacted organ due to rhizomania. Rhizomania infected roots were obtained by baiting for the disease from field soil, and as a control healthy roots grown on non-field soil. Firstly, RT-RPA was performed at isothermal conditions using total RNA isolated from rhizomania baited roots, and healthy roots along with a no template control. To determine sensitivity of the CRISPR-Cas12a based assay, these RT-RPA reactions were serially diluted ten-folds and used 5 µL from dilutions in the CRISPR-Cas12a reaction that contains fluorescently labelled ssDNA reporter. After incubating at 37°C, and the results revealed dramatic strong fluorescence signal in the reactions that had RT-RPA template from rhizomania infected roots compared to the signal obtained for reactions with healthy root samples (Fig. 2). Signal observed with no template control was considered background, and this value was subtracted from the signals that were obtained for rhizomania containing roots and healthy roots samples to alleviate background noise accompanying with the reagents. A linear correlation of signal reduction with increasing folds of serial dilution was observed and limit of sensitivity was 0.1 ng concentration (Fig. 2). Of note, the field soil tested here showed positive for rhizomania in a different experiment using ELISA (data not shown). In summary, we have developed an isothermal RT-RPA based CRISPR-Cas12a diagnostic method to detect BNYVV in rhizomania infected roots of sugarbeet.

In conclusion, we present a CRISPR-Cas based method for detecting BNYVV in roots of sugarbeet. We first developed one-step isothermal RT-RPA method for BNYVV detection from rhizomania infected sugarbeet roots. The RT-RPA method is simple, and isothermal as oppose to regular RT-PCR assays. Subsequently, we have developed CRISPR-Cas12a based detection method for BNYVV, which has set the stage for sensitive, specific, and high throughput detection platform for rhizomania evaluation. The development and validation of CRISPR-based BNYVV diagnostic method for sugarbeet roots has advantageous in terms of providing sensitivity and robustness at isothermal condition and hence, would serve as a valuable tool for sugarbeet industries for evaluating viruses for driving disease management strategies. Moreover, this technology developed for virus diagnostic for underground

root-tissue can be applied for setting up CRISPR-based detection platform for other crop infecting viruses including soil-borne disease-causing agents.

Figure 1. Rhizomania baiting and RT-RPA mediated detection of BNYVV from *B. vulgaris*. (A) Whole plant pictures of *B. vulgaris* baited for rhizomania under greenhouse condition in pots using infected field soil six weeks post inoculation. (B) Representative individual plants showing root phenotype associated with rhizomania. Extreme care was taken to gently remove the soil from root. (C) and (D) Detection of RT-RPA amplicon on agarose gel. Even 5 uL (C) and 10 uL (D) loading volume, show strong visual band from a 50 uL reaction. Lanes: infected refers to sugarbeet roots baited for rhizomania disease from field soil. Healthy refers to root obtained from sugarbeet grown in potting mix used for growing plants under laboratory conditions. NTC stands for no template control, and M stands for size marker.

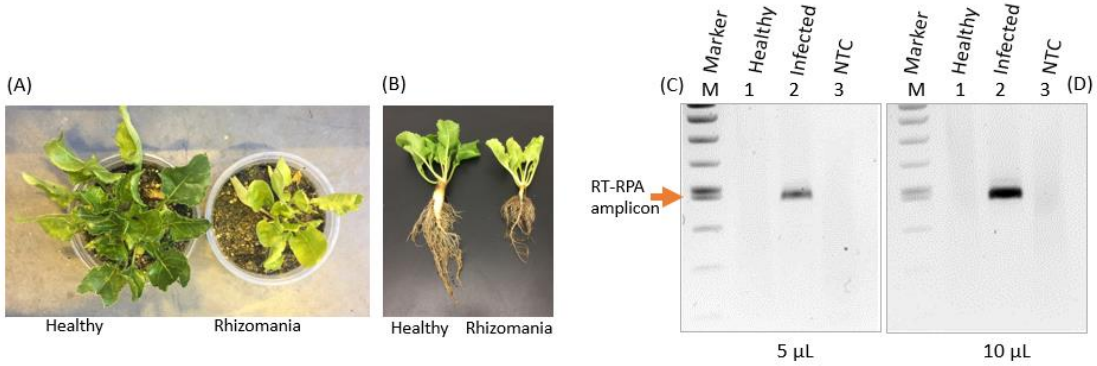

Figure 2. CRISPR-cas12 based detection of BNYVV in root tissue of *B. vulgaris* baited for rhizomania using infected field soil. The template DNA used in this assay was obtained through RT-RPA from rhizomania-infected and healthy roots of sugarbeet. To determine the limit of detection, the RT-RPA product was diluted subjected to CRISPR-Cas12a reporter assay as described in the Materials and Methods. No template reaction serves as the negative control and used for background subtraction. Values plotted on the Y-axis represent background subtracted fluorescence. Whereas X-axis represents dilution concentrations of the RT-RPA generated template DNA. Error bars represents standard deviation (STDEV) on replicates (*n=3*).

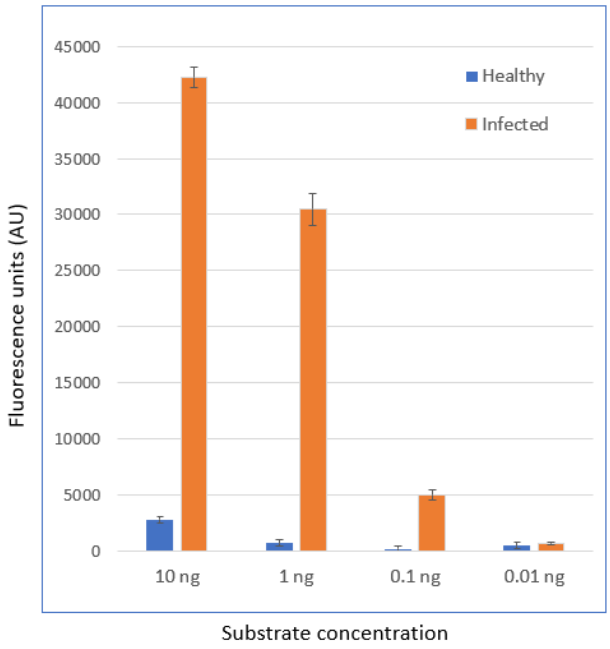

Table.1. Sequences of primers, reporter, CRISPR-guide RNA, and target used in this study. Coordinates relative to target BNYVV RNA-1 are indicated in parenthesis.

Name	Sequence
VR-1	5'-gcgttctgattatcagaatcaacgagttggtg-3' (3064 – 3095)
VR-2	5'-atatgttcaccagtctcatcggaataatgaatg-3' (3528 - 3496)
VR-3	5'-cgttctgattatcagaatcaac-3' (3065 – 3086)
VR-4	5'-atatgttcaccagtctcatcg-3' (3528 – 3508)
Reporter	/56-FAM/TTATT/BHQ-1
BNY-gR1	5'-UAAUUUCUACUAAGUGUAGAUCAGCCUAUGAUUGGCGGUUGC-3' (3180 – 3200)
BNYVV synthetic template DNA (GenBank accession number MT227164)	gcgttctgattatcagaatcaacgagttggtgatgagcttctttcttgggactttcacacgcctcacaaagcattggatgttactggtaagcaaattatttttgttgatgagtttacagcctatgattggcggttgctagctgtgttggcttatagaaatcatgcccatactatttacttagttggtgatgagcagcagactggtattcaagagggtcgtggagaaggaatatcgatacttaacaaaattgatctgtctaaggtttctacacatgttccaatcatgaactttagaaatcctgtccgtgatgttaaggtattaaattatctgttcgggtctcgtatggttcctatgtcttccgttgaaaagggatttagtttcggggatattaaagaattttcgtctttgtcaaatatcccagacactaaaatcattcattattccgatgagactggtgaacatat (3064 –3528)

Acknowledgements

We thank Jonathan Neubauer for technical support with Tecan plate reader, Alyssa Flobinus for general laboratory support, and Alexander Dessert for plant care. We greatly acknowledge Mark Bloomquist, Southern Minnesota Beet Sugar Cooperative for providing field soil for rhizomania analysis. This research was supported by the U. S. Department of Agriculture, Agricultural Research Service. Any commercial products mentioned in this article is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the U. S. Department of Agriculture.

References

1. Rush, C. M., Liu, H. Y., Lewellen, R. T., and Acosta-Leal, R. (2006). The continuing saga of rhizomania of sugar beets in the United States. Plant Dis. 90: 4-15.
2. Chen, J. S., MA, E., Harrington, L. B., Costa, M. D., Tian, X., Palefsky, M. J., and Doudna, J. A. (2018). CRISPR-Cas12a target binding unleashes indiscriminate single-stranded DNase activity. Science. 360: 436.
3. Torrance, L., Pead, M. T., and Buxton, G. (1988). Production and some characteristics of monoclonal antibodies against beet necrotic yellow vein virus. Ann. Appl. Biol. 113, 519–530.
4. Weiland, J. J., Sharma-Poudel., R., Flobinus, A., Cook, D. E., Secor, G. A., and Bolton, M. D. (2020). RNAseq analysis of rhizomania-infected sugar beet provides the first genome sequence of beet necrotic yellow vein virus from the USA and identifies a novel alphanecrovirus and putative satellite viruses. Viruses. 12: 626.