DEVELOPMENT OF A NEW METHOD FOR RAPID ISOLATION AND IDENTIFICATION OF RESISTANT BREAKING STRAINS OF BNYVV FROM FIELD SOILS

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INTRODUCTION

Recent studies in our lab revealed that although a number of BNYVV genotypes are able to overcome genetic resistance in sugar beets not all of those are environmentally fit and may not survive, but those with a VL amino acid motif in the hypervariable region of BNYVV RNA 3 are fit and will survive in the soil for years. The only way to determine what RB strains are present in a field is to bait the virus from the soil and sequence it. Although sequencing is getting cheaper, it is still a method that not many labs can easily do so an improved method for detecting BNYVV in the soil and determining the virus haplotype was need. With this need in mind, studies were initiated to develop a new method of testing field soils for the presence of BNYVV and evaluating High Resolution Melting Analysis (HRM) for its ability to differentiate WT and RB strains of BNYVV from field soils.

MATERIALS AND METHODS

With traditional methods, it takes approximately eight weeks to bait BNYVV from field soils and another three to four weeks to determine the strain of BNYVV by traditional sequencing techniques. A new method of baiting BNYVV was developed that is based on the fact that the soilborne fungus that transmits BNYVV in the soil, i.e. *Polymyxa betae,* produces zoospores that are released when soils are flooded and then swim to host roots where they penetrate the root and transmit the virus. Field soils collected from grower's fields were taken to the lab and tested for the presence of BNYVV. To serve as a positive control, one soil was amended with dried roots from a plant exhibiting classic symptoms of rhizomania. Soils were placed in petri dishes, wet to near field capacity and incubated at 82F for 24hr. After the initial hydration stage, the damp soils were flooded and sugar beet seedling from cultivars susceptible to BNYVV, or with *Rz1*or *Rz2* resistance genes, were placed in the petri dishes, incubated at 82F and allowed to float in the water for an additional 24 hr. During this period, it was anticipated that zoospores of *P. betae* would be attracted by root exudates and would mass on the seedling root and initiate the infection process. After the 24hr float/infection period, seedlings were collected and total nucleic acids extracted. Specific PCR primers designed to target the hypervariable region on BNYVV RNA 3 were used for c-DNA synthesis and rt-qPCR for detection of BNYVV.

In preliminary tests, HRM was used to screen a number of archived RB and WT BNYVV isolates collected over the last 10 years. With this method, the hypervariable region of P25 BNYVV RNA 3 is amplified in a reaction containing a double-strand DNA (dsDNA)–binding dye, which fluoresces brightly only when bound to dsDNA. After the PCR, the product is again heated in the ABI thermocycler and the double stranded PCR product begans to melt. When this happens, the dye is released, causing a change in fluorescence that results in a unique melting curve profile for each particular BNYVV strain. To verify that each sample and resulting melt curve represented a specific WT or RB sequence of BNYVV, products were submitted to Gene Technologies Laboratory, College Station, Texas for sequencing. In this way, individual melting curves were associated with WT and specific RB BNYVV genotypes. Following these preliminary tests, tissue samples from the seedlings used to bait BNYVV from field soils were collected and total RNA was purified. Samples that tested positive for BNYVV were then used in HRM analysis.

RESULTS AND DISCUSSION

The seedling baiting method was successful in isolating BNYVV from the amended soils. Incubating the damp soil at 27 C for 24 hours "primed" the sporosori in the rhizomania roots and soil, and when the soils were flooded for 24 hours the sporosori rapidly released zoospores of *P. betae.* The zoospores were attracted to the sugar beet seedling, which they infected and subsequently transmitted BNYVV. The high sensitivity of rt-PCR allowed detection of BNYVV in the bait plants after the 24 hour incubation period, whereas the low initial titer in the seedlings would not have been detectable by ELISA that is often used with the traditional method of BNYVV detection. The ct values for the majority of bait seedlings ranged from 28 - 34, indicating clear amplification of BNYVV. When baiting tests were conducted on non-amended field soils BNYVV was detected with ct values ranging from 28 - 37. However, not all field soils tested positive and when a traditional 8 week baiting test was used BNYVV was detected in some, indicating that the rapid baiting method may not be as sensitive as the longer traditional test where BNYVV has an opportunity to multiply in the infected seeding. In a final test in which seedling with *Rz1* or *Rz2* or no resistance to BNYVV were used as bait plants, the susceptible controls with no genetic resistance had the highest

BNYVV titers and ct values of 28 - 29. The majority of seedlings with either *Rz1* or *Rz2* resistance had much lower titers and ct values that ranged from 32 - 36. However, there was no indication in these preliminary studies that use of a resistant cultivar as the bait seedling preferentially selected or excluded specific haplotypes of BNYVV.

The second objective of this study was to evaluate the application of HRM analysis for differentiating WT and RB strains of BNYVV. Archived samples of WT and RB cDNA from field isolates of BNYVV were used as positive controls in this study, and HRM analysis was able to clearly differentiate WT and RB strains (Fig. 1). It was also able to differentiate an archived strain designated as CIV from both WT and RB. The CIV strain was isolated from cultivars with *Rz1* resistance but unlike the RB strains, the CIV strain did not cause symptoms of rhizomania. After these preliminary tests, HRM was used to evaluate isolates of BNYVV obtained from grower's fields, using the rapid baiting technique. HRM analysis identified and differentiated both WT and RB strains of BNYVV and revealed that RB strains are predominant in Southern Minnesota, while in the northern American Crystal area there is more of a mix of WT and RB strains. It was not unusual to find WT and RB strains in the same field and this finding supports previous studies that revealed an aggregated distribution of RB strains in many fields. This complicates studies by sugar beet seed breeding companies trying to establish research plots in fields that were previously affected by RB strains of BNYVV. HRM primers developed for the hypervariable region of BNYVV were specific to BNYVV and did not amplify BSBMV.

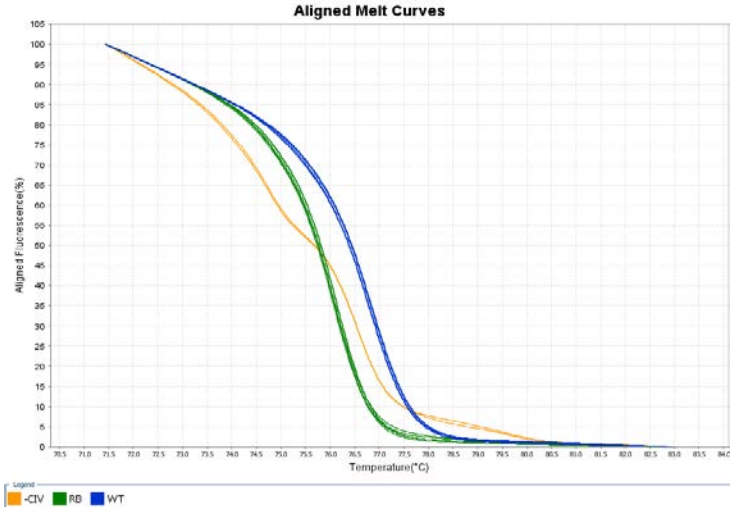

Figure 1. High resolution melting cures for strains of BNYVV. HRM was able to differentiate between WT, CIV and RB strains of BNYVV.

In conclusion, HRM analysis used in combination with the rapid baiting technique has potential to significantly improve our ability to rapidly detect and identify RB strains of BNYVV in grower's fields. Compared with traditional ELISA-based techniques, the zoospore baiting-PCR method reduced time of BNYVV detection in field soils from 8 weeks to three days. However, if BNYVV titer in infested soils is very low the longer traditional baiting method might be required to amplify BNYVV to a level where it can be detected by PCR. HRM analysis provided a rapid, sensitive and accurate method to detect and identify different strains of BNYVV in plant and soil samples. Zoospore baiting plus HRM can benefit growers by revealing RB strains of BNYVV in fields before planting and can benefit breeders and seed companies by identifying fields with RB strains of BNYVV and revealing how breeding lines respond to infection by specific virus genotypes. Furthermore, HRM should be exceptionally useful in studies of BSBMV and BNYVV field population genetics.