Development and Validation of High-Resolution Melting Markers Derived from *Ry_{sto}* STS Markers for High-Throughput Marker-Assisted Selection of Potato Carrying *Ry_{sto}*

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ABSTRACT

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Sequence analysis of the chromosome region harboring the sequence-tagged site (STS) markers YES3-3A and YES3-3B for *Ry_{sto}*, a gene responsible for extreme resistance to *Potato virus Y* (PVY) in potato, was performed in tetraploid potato 'Barbara' (*Rrrr*) and 'AC Chaleur' (*rrrr*) as well as their progeny selections. Three and two sequence variants were identified in Barbara resistant (R) selections and AC Chaleur susceptible (S) selections, respectively. Further analysis indicates that the variant with a 21-nucleotide (nt) deletion is likely the chromosome copy harboring the STS markers. Two primer pairs, one targeting the region containing a

20-nt deletion and the other targeting the region anchoring the YES3-3A reverse primer, were designed. As anticipated, pair one produced two visible fragments in Barbara-R bulk and one visible fragment in AC Chaleur-S bulk; pair two produced one visible fragment in all samples. When subjected to high-resolution melting (HRM) analysis, two distinct melting profiles for R and S samples were observed. Analysis of 147 progeny of Barbara × AC Chaleur revealed 72 and 75 progeny with R and S melting profiles, respectively, which was consistent with YES3-3A and YES3-3B assays and phenotyping analysis, thus demonstrating the potential of HRM profiles as novel molecular markers for *Ry_{sto}*. The efficacy of the newly developed HRM markers for high-throughput marker-assisted selection for *Ry_{sto}*-conferred resistance to PVY was validated further with three populations involving Barbara as the R parent.

Potato virus Y (PVY; genus *Potyvirus*, family *Potyviridae*) is one of the most agriculturally important pathogens worldwide, affecting potato as well as several other important crops belonging to the nightshade family (Singh et al. 2008). Many strains and substrains, including the ordinary strain (PVY^O), the tobacco vein necrosis strain (PVY^N), and several PVY^O-PVY^N recombinant strains such as PVY^{N:O} (or PVY^N-Wilga) and recombinant PVY^{NTN} (Eu-PVY^{NTN} and PVY^{NTN-NW}) have been characterized (Karasev and Gray 2013; Nie et al. 2013). In potato, due to the emergence of the recombinant strains or isolates which normally cause mild symptoms in most commonly grown cultivars, and the increased production of susceptible cultivars that do not express clear visual symptoms, thus evading symptom-based roguing and field inspections, an increase of PVY incidence in potato crops has been observed in North America over the last decade (Karasev and Gray 2013; Nie et al. 2012, 2013). The use of clean seed tubers to reduce PVY inoculum and the frequent application of mineral oil to interfere with aphid-mediated PVY transmission are common practices employed in potato production systems, especially seed potato production, to minimize the impact of PVY on potato yield and quality. However, these practices incur significant costs to growers (MacKenzie et al. 2014).

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The use of the resistant cultivars offers one of the most effective means for management of PVY and PVY-caused diseases in potato as well as other Solanaceous crops (Karasev and Gray 2013; Singh et al. 2008). In potato, two types of host resistance—namely, hypersensitive resistance (HR) conferred by *N* genes and extreme resistance (ER) conferred by *R* genes—have been recognized against PVY (Cockerham 1970; Nie et al. 2015, in press; Singh et al. 2008). Although most *N* genes are strain specific (Jones 1990; Nie et al. 2015; Singh et al. 2008), *Ny-1* and *Ny-2* are reported to be strain nonspecific (Szajko et al. 2008, 2014). Resistance (*R*) genes, including *Ry_{adg}*, *Ry_{sto}*, and *Ry_{chc}*, which were derived from *Solanum tuberosum* subsp. *andigena*, *S. stoloniferum*, and *S. chacoense*, respectively, are PVY strain nonspecific. These *Ry* genes have been integrated into potato germplasm and are being used for breeding potato cultivars with ER against PVY (Fulladolsa et al. 2015; Mori et al. 2011, 2012; Ottoman et al. 2009). Undoubtedly, the development of cultivars possessing one of the *Ry* genes, as well as other agronomic or economically important traits, is highly desirable.

The use of molecular markers for the marker-assisted selection (MAS) of cultivars with desirable traits has proven to be time and cost efficient in plant breeding (Xu and Crouch 2008). Several markers tightly linked to various *Ry* genes have been developed. These markers include the sequence-tagged site (STS) markers YES3-3A and YES3-3B as well as a cleaved amplified polymorphism marker GP122 for *Ry_{sto}* on chromosome 12 (Flis et al. 2005; Song and Schwarzfischer 2008; Valkonen et al. 2008), the sequence-characterized region marker RYSC3 for *Ry_{adg}* on chromosome 11 (Kasai et al. 2000), and the random amplified polymorphic DNA marker 38-530 and STS markers Ry186 and Ry364 for *Ry_{chc}* on chromosome 9 (Hosaka et al. 2001; Mori et al. 2011, 2012; Sato et al. 2006).

Ry_{sto} has been integrated into various potato cultivars, including 'Barbara' (Barker 1996; Song and Schwarzfischer 2008), and the STS markers YES3-3A and YES3-3B were found to cosegregate with *Ry_{sto}* (Kuhl 2011). In our previous studies, Barbara and its three advanced selections were found to be ER to PVY and to possess both YES3-3A and YES3-3B markers (Nie et al. in press). However, these gel electrophoresis-based markers are inefficient because only a small number of samples can be processed in each gel. Moreover, the markers are difficult to use because the target marker amplicon, especially the YES3-3B, and these primers generate nonspecific amplicons close in fragment size to the target product. Consequently, a polyacrylamide gel or a high-concentration (>2.0%) agarose gel is required to separate the marker from other amplicons (Fulladolsa et al. 2015; Song and Schwarzfischer 2008).

The objectives of this research were to (i) identify the *Ry_{sto}*-linked single-nucleotide polymorphisms (SNP) in the chromosome region harboring the YES3-3A and YES3-3B markers; (ii) based on the SNP, develop nongel-based high-resolution DNA melting (HRM) markers that are tightly linked to *Ry_{sto}*; and (iii) validate the efficacy of the newly developed HRM markers for high-throughput selection for *Ry_{sto}*-mediated ER to PVY in potato breeding.

MATERIALS AND METHODS

Plant materials, PVY isolates, and virus-inoculation. Potato cultivars and breeding clones Barbara, 'AC Chaleur', 'Sunrise', 'N1503-19', and 'G7815-9Y', as well as segregating populations of Barbara × AC Chaleur (*n* = 147), Barbara × Sunrise (*n* = 65), Barbara × N1503-19 (*n* = 64), and Barbara × G7815-9Y (*n* = 62), were used in this study. The clones were maintained at the Fredericton Research and Development Centre, Agriculture and Agri-Food Canada, Fredericton, Canada, in the greenhouse with a cycle of 16 h of light and 8 h of darkness. The natural light was supplemented with artificial light or shade to give a light intensity of approximately 90 μm²/s. The temperature was set at 24 and 18°C (day and night, respectively), with a humidity of 75%. The potato clones were assessed for their response to PVY infection by mechanical and graft inoculations with PVY^O prior to being used as breeding parents, as described by De Jong et al. (2001). Plants that could not be infected by PVY even after graft inoculation are considered to have ER. The clones and cultivars were also assessed for their response to different PVY strains, including PVY^O (isolate FL), PVY^N (isolate Jg), PVY^{N:O} (isolate Mb58), and PVY^{NTN} (isolate SI), by both mechanical and graft inoculations, as described previously (Nie et al. 2015) to verify their susceptibility (S) or resistance (R) to PVY. In addition, several advanced selections derived from Barbara, including the PVY-R selections F00069, F08086, and F08087 and PVY-S selections F12080 and F12081, were included for SNP detection and HRM marker development.

Seed of Barbara (R) × AC Chaleur, Sunrise, N1503-19, or G7815-9Y (S) were sown in flat trays filled with Pro-Mix potting soil (Premier Horticulture Inc., Rivière-du-Loup, Quebec, Canada) for germination in a greenhouse, and the resulting seedlings were transplanted to 5-in. (12.7-cm) pots containing Pro-Mix potting soil. When the seedlings reached approximately five-leaf stage, one leaf was taken from each plant for genomic DNA extraction. Shortly after that, the plants were inoculated with PVY^{NTN} by graft inoculation (progeny of Barbara × AC Chaleur) or by mechanical wounding (progeny of Barbara × Sunrise, N1503-19, or G7815-9Y).

Enzyme-linked immunosorbent assay. Enzyme-linked immunosorbent assay (ELISA) with PVY polyclonal antibodies (Adgen Phytodiagnosics-Neogen, Scotland, UK) was performed according to the manufacturer's instructions. A sample was considered positive when absorbance at 405 nm (*A*₄₀₅) was three times of the negative (healthy) control with a reading ≥ 0.100 (Nie et al. 2015). Samples whose *A*₄₀₅ fell between the criteria (i.e., ≥3× the healthy control and <0.100) were retested by ELISA.

DNA extractions. Genomic DNA was isolated using the Qiagen DNeasy Plant Mini column purification kit protocol (Qiagen,

Frederick, MD). Young leaf tissues (approximately 100 mg) taken from greenhouse-grown plants were homogenized with 550 μl of AP1 lysis buffer containing 1.0% (wt/vol) RNaseA, and lysed using Lysing Matrix A with FastPrep-24 (MP Biomedicals, Eschwege, Germany), 450 μl of lysate was applied to the Qiagen shredder column, and, subsequently, the manufacturer's protocol was followed. The final elution step was performed with the provided elution buffer at a volume of 100 μl. Following DNA extraction, the quality and quantity of DNA was analyzed using the Qubit Fluorometer (Invitrogen, Carlsbad, CA) and NanoDrop 2000 Spectrophotometer (ThermoFisher Scientific, Waltham, MA). The concentration of the DNA was adjusted to 10 ng/μl.

Primer design. For cloning the chromosome region harboring the YES3 markers, the YES3-3B STS sequence (GenBank accession BV725478) was used for BLAST with the reference genome sequence of *S. tuberosum* group Phureja DM1-3 (v4.04, http://potato.plantbiology.msu.edu/integrated_searches.shtml). The most similar reference sequence (chr12_59061324-59062403) was padded with approximately 400 bp at each end and then compared with various data sets using BLAST at the Potato Genomics Resources website (<http://solanaceae.plantbiology.msu.edu/blast.shtml>), which resulted in seven sequences with high similarity to the YES3 STS sequence. These sequences are tomato chr12 (SL2.40ch12)_64170541-64170853, tomato chr12 (SL2.40ch00)_15671646-15670855, *S. commersonii* cultivar cmm1t_JXZD01046435_9243-8161, *S. tuberosum* BAC chromosome 12_JF317595_128184-129238, *S. tuberosum* BAC chromosome 12_JF317598_54167-55221, *S. tuberosum* BAC chromosome 12_JF317621_45762-46816, and *S. tuberosum* BAC chromosome 12_JF317622_47555-48609. After aligning the sequences, a primer pair (forward, YES3-F1, 5'-ATGGACAATGATCTTATTCAAG, and reverse, YES3-R2, 5'-TTCCATGTGCTCTTGATGCTA-3') that was predicted to produce a fragment of approximately 653 bp was designed.

After the sequences harboring the YES3 markers were obtained from Barbara (R), AC Chaleur (S), and the R and S pools from Barbara × AC Chaleur progeny, the candidate SNP linked to *R* genes were predicted according to the sequence variant ratio. Thereafter, primers suitable for production of amplicons containing the SNP between the R and S genotypes were designed for HRM analysis. These primers were set 1, YES_F182 (5'-CAGGGTATTGGAAATATTAGTGGA-3') with YES_R345 (5'-CATGTGCGTAATACAAGAAGC-3'), for which the predicted amplicon sizes were 164 bp for R alleles and 184 and 189 bp for S alleles; and set 2, YES_F393 (5'-CAAAGGCAATCTCATGAAAGC-3') with YES_R563 (5'-TCCTAATGATTCCCGTGAGG-3'), for which the predicted amplicon sizes for R and S alleles were 171 and 172 bp, respectively.

Regular polymerase chain reaction and gel electrophoresis. Regular polymerase chain reaction (PCR) with appropriate primer sets and DNA template was carried out on a C1000 Touch Thermal Cycler (Bio-Rad Laboratories, Hercules, CA). The 25-μl PCR mix for each reaction contained 1× PCR buffer, 0.2 mM each dNTP, 0.5 mM each primer, 0.25 μl of Taq or Q5 DNA polymerase, and 20 ng of genomic DNA. A 35-cycle PCR was performed, commencing with an initial denaturation step of 95°C for 105 s, followed by 35 cycles of 45 s at 94°C, 45 s at 60°C, and 60 s at 72°C. A final elongation step of 72°C for 5 min was then performed. After the reaction, 10 μl of PCR products was loaded onto a 1.5% agarose gel (2.2% agarose gel for the PCR products of YES3-3B marker) stained with Gel Red (Biotium Inc., Hayward, CA). The presence or absence of the predicted amplicons were visualized and recorded with BioSpectrum Imaging System (UVP Inc., Upland, CA).

Cloning and sequencing the chromosome region harboring the YES3 markers. PCR products of YES3-F1 and YES3-R2 amplified with Q5 High-Fidelity DNA Polymerase (New England Biolabs, Ipswich, MA) were purified using MinElute Gel Extraction Kit (Qiagen) after agarose gel electrophoresis. The purified amplicons were cloned into the Zero Blunt TOPO PCR Cloning Kit (ThermoFisher Scientific). Approximately 20 clones from each of the four samples (Barbara, AC Chaleur, R pool, and S pool) were sequenced at

Genome Quebec (Montreal, Quebec, Canada) with the Sanger method using M13 forward and reverse sequencing primers.

Real-time PCR and HRM analysis. In all, 30 ng of DNA template was used in the 10- μ l reaction, along with 5 μ l of the 2 $\times$ Precision Melt Supermix containing EvaGreen dye (Bio-Rad Laboratories) and 0.2 mM each primer. The real-time PCR optimization was carried out using a 45-step amplification, each beginning with 95°C for 10 s, a gradient annealing temperature of 56 to 68°C for 30 s, followed by 72°C for 30 s. The optimum annealing temperature of 60°C was determined based on the lowest cycle threshold value and specific amplification. Next, DNA samples were grouped into either R or S pools and were used to test our primer combinations for their efficacy in genotyping samples; plants were separated into these categories via YES3-3A markers and ELISA results.

Following the establishment of suitable melt profiles for each genotype, each sample was evaluated individually with HRM analysis on multiple occasions to verify the reproducibility of the assay. The primer pairs used were YES_F182 with YES_R345 and YES_F393 with YES_R563. Real-time PCR conditions were followed as above using the CFX96 Real-Time System (Bio-Rad Laboratories), with a final extension of 72°C for 6 min. The melt profile was determined by a 95°C denaturation step for 60 s prior to annealing at 55°C to ensure uniform annealing of all amplicons and then increasing the temperature by 0.2°C increments to 95°C, with a 10-s plate read at each temperature increase. The DNA melting profile was also performed on a 96-well LightScanner HR|96 (Idaho Technology Inc., Salt Lake City, UT) after 10 μ l of mineral oil was overlaid to each sample. HRM conditions were identical to those described before (55 to 95°C); however, plate reads were taken at 0.1°C increments, allowing further resolution. The melting data obtained from CFX96 and LightScanner were analyzed using Precision Melt Analysis Software (Bio-Rad Laboratories) and LightScanner Software v2.0 (Idaho Technology), respectively.

RESULTS

PVY resistance in Barbara. Barbara has been demonstrated to have ER to PVY conferred by *Ry_{sto}* (Barker 1996; Song and Schwarzfischer 2008; Song et al. 2005). Indeed, mechanical inoculation of Barbara plants with four PVY isolates belonging to

PVY^O, PVY^N, PVY^{N:O}, and PVY^{NTN} was unable to infect the plants. Neither visible symptoms nor ELISA-detectable levels of PVY were observed. Top-graft inoculation of the plants with the PVY isolates incited necrotic spots on newly emerging leaves, commencing approximately 10 days postinoculation; however, the virus level remained too low to be detected by ELISA (Fig. 1). Progeny plants from tubers of the symptomatic PVY graft-inoculated plants were healthy in appearance and PVY free. These results confirm that the resistance to PVY in Barbara is, indeed, ER and strain nonspecific. On the other hand, AC Chaleur, Sunrise, N1503-19, and G7815-9Y were infected with the tested PVY strains after either mechanical or graft inoculation, demonstrating their susceptibility to PVY infection. Consistent with the phenotype, the STS markers YES3-3A and YES3-3B were present in Barbara but absent in AC Chaleur, Sunrise, N1503-19, and G7815-9Y (data not shown).

A segregating population comprising 147 progeny of Barbara $\times$ AC Chaleur were graft inoculated with PVY^{NTN}, then subjected to ELISA testing. Of the 147 plants, 72 were determined as R and 75 as S to PVY. The segregation ratio of R/S = 72:75 for ER in this family fits a 1:1 segregating ratio ($\chi^2 = 0.061224$, $P = 0.8046$), a ratio that can be expected from a simplex (*Rrrr*) $\times$ nulliplex (*rrrr*) cross. This result is consistent with that in Barbara reported by Barker (1996).

SNP linked to *Ry_{sto}* in chromosome region harboring the STS marker YES3. Although the STS sequence YES3-3B RyEM3-3 (GenBank accession number BV725478) from 'Assia' (ER to PVY, conferred by *Ry_{sto}*) was available (Song and Schwarzfischer 2008), the SNP associated with *Ry_{sto}* are unclear. Sequence analysis of the chromosome region in which the YES3-3A and YES3-3B markers are embedded reveals three variant groups from 20 clones in Barbara (Fig. 2): group 1 comprised 6 entries/clones, group 2 comprised 5 entries/clones, and group 3 comprised 9 entries/clones. Variant 1 (V1, accession number KX434791) was 21 and 26 nucleotides (nt) shorter than variant 2 (V2, accession number KX434792) and variant 3 (V3, accession number KX434792), respectively; and V2 was 5 nt shorter than V3. In AC Chaleur, two variant groups, one 5 nt shorter than the other, were found. In the R pool (F00069, F08086, and F08087) and the S pool (F12080 and F12081), three and two sequence variant groups were found, respectively. Further comparison of the sequences revealed that the shortest variant (V1) was present in Barbara and the R pool but absent in AC Chaleur and the S pool, whereas V2 and V3 were present in all samples. The respective V1:V2:V3 ratio in Barbara and R pool was 6:5:9 and 6:7:12, respectively, both fitting a 1:1:2 ratio ($\chi^2 = 0.3$, $P = 0.8607$ for Barbara and $\chi^2 = 0.12$, $P = 0.9418$ for the R pool). Considering the fact that a simplex *Ry_{sto}* in Barbara confers the resistance, it is most likely that the YES3-3A and YES3-3B markers are harbored by V1. In silico PCR analysis revealed that the YES3-3A primers can lead to amplification of a 341-bp fragment from V1 in Barbara and the R pool. The YES3-3B primers, on the other hand, can lead to amplification from all the sequence variants. In Barbara and the R pool, three fragments (284 bp from V1, 304 bp from V2, and 309 bp from V3) are expected to be amplified by YES3-3B primers. In AC Chaleur and the S pool, two fragments (304 and 309 bp) are anticipated to be produced by the primer set. Taken together, the results demonstrate that the V1, designated as *V_R*, in Barbara and its R progeny harbors the YES3-3A and the YES3-3B markers. The nonmarker-bearing variants V2 and V3 are designated as *V_{r1}* and *V_{r2}*, respectively. Alignment of the sequence variants from Barbara and the R pool revealed that, for YES3-3A, the SNPs are located in the reverse primer and, for YES3-3B, the polymorphisms are the 21-nt deletions. This also explains why a high-concentration agarose gel or a polyacrylamide gel is needed to detect the YES3-3B fragment correctly (Fulladolsa et al. 2015; Nie et al. in press; Song and Schwarzfischer 2008).

Development of HRM assay for detection of *Ry_{sto}*-bearing selections. Two primer pairs were designed to differentiate the

Fig. 1. Response of Barbara potato to *Potato virus Y* (PVY) (strain NTN) after graft inoculation. Enzyme-linked immunosorbent assay (ELISA) testing was performed to detect PVY. The PVY-infected scion (inoculum) was 'Russet Norkotah'. Photo was taken 16 days after graft inoculation.

amplicon of V_R , which harbors the YES3-3A and YES3-3B markers, from that of other variants because of different melting profiles. The primer pair YES_F182 and YES_R345 covers the region where a 20-nt deletion occurs, whereas the pair YES_F393 and YES_R563 covers the region where the YES3-3A-associated SNP are located. PCR with the primers produced fragments as predicted in Barbara, AC Chaleur, the R pool, and the S pool (Figs. 3A and 4A). When the PCR products were subjected to HRM assays, distinct melt profiles between those of Barbara-R pool (R profile) and those of AC Chaleur-S pool (S profile) were observed (Figs. 3B, 3C, 4B, and 4C). For amplicons produced by YES_F182/YES_R345, a difference in melting temperature was observed between approximately 81 and 82°C in AC Chaleur-S pool and approximately 80°C in Barbara-R pool (Fig. 3B and C), likely due to the nucleotide deletions in the V_R . For amplicons produced by YES_F393/YES_R563, a small but distinct peak at approximately 72.5°C occurred in amplicons of Barbara-R pool (R profile) but not in the AC Chaleur-S pool (S profile) (Fig. 4B and C).

Testing the candidate markers in a segregating population of Barbara × AC Chaleur. To test whether the differential melting profiles of the amplicons were associated with resistance or susceptibility and, moreover, whether they can be used as markers for R_{ysto} in potato breeding, the segregating progeny of Barbara × AC Chaleur were analyzed. Of the 147 progeny, 72 and 75 exhibited R and S melting profiles, respectively, with amplicons produced by YES_F182/YES_R345 (Fig. 5). Despite a slight difference in the melting peaks in two detection systems (i.e., CFX96 and Light-Scanner), both gave identical calls. For amplicons produced by YES_F393/YES_R563, 59 seedlings exhibited melting profiles that featured the small but distinct melting peak at 72.5°C (Fig. 6A and B).

Further examination of the melting profiles of all samples revealed that 13 samples exhibited a distinct melting peak at approximately 80°C (Fig. 6, lines in blue). Surprisingly, these 13 samples exhibited R melting profiles in the YES-F182/YES-R345 HRM assay and, thus, the distinct melting peak at approximately 80°C exhibited by these samples with the YES_F393/YES_R563 assay can also be considered an R melting profile. When both R melting profiles are combined, the YES_F393/YES_R563 HRM assay produced results identical to that of the YES-F182/YES-R345 assay.

The gel-based YES3-3A and YES3-3B markers were also tested in the population. Consistent with the HRM assays, the 72 plants that showed R melting profiles tested positive for both YES3-3A and YES3-3B, and the remaining 75 plants that exhibited S melting profiles tested negative for the markers. Moreover, the results are in complete (100%) agreement with the phenotyping results: plants that contained the markers (YES3-3A and YES3-3B) and R melting profiles were resistant to PVY, whereas plants that did not possess the markers and R melting profile were susceptible to PVY (Supplementary Table S1). The R profiles, therefore, can be used as markers for R_{ysto} , and were designated as YES-HRM1 and YES-HRM2, respectively.

Validation of the newly developed HRM markers for robust MAS for R_{ysto} . Three populations involving Barbara (Barbara × Sunrise, Barbara × G7815-95, and Barbara × N1503-19) were used to assess and validate the efficacy of YES-HRM1 and YES-HRM2 as markers for efficient selection of plants carrying R_{ysto} . Of the 65 progeny of Barbara × Sunrise, 34 tested positive (i.e., showing R melting profile) for both YES-HRM1 and YES-HRM2, and 31 negative (i.e., showing S melting profile)

Fig. 2. Sequence variants of the chromosome 12 region harboring the sequence-tagged site markers YES3-3A and YES3-3B in Barbara potato. On the left is the phylogenetic grouping of all sequence entries (clones) and on the right is the alignment of sequence variants 1 to 3. The GenBank accession numbers of the sequence variants are KX434791, KX434792, and KX434793.

B

C

Fig. 3. Post-polymerase chain reaction analysis of amplicons resulting from primer pair YES_F182 and YES_R345 in Barbara and AC Chaleur potato as well as DNA pools of *Potato virus Y* (PVY)-resistant (R) or -susceptible (S) progeny selections from Barbara. **A**, Agarose gel electrophoresis. Lane M, 1-kb Plus DNA Ladder (Invitrogen). Sizes of the ladder DNA of 100 to 500 bp are shown on the left side of the picture. Amplicon sizes are indicated on the right of the picture. **B**, High-resolution DNA melting analysis carried out on LightScanner with respective software. The upper panel is the normalized melting curve data plotted with the normalized fluorescence against temperature (°C) and the lower panel is the normalized melting peaks with the normalized δ fluorescence versus temperature (°C). **C**, High-resolution DNA melting analysis carried out on CFX96 using the Precision Melt Analysis software. The upper panel is the melt curve data plotted as relative fluorescent units (RFU, 10^3) against temperature (°C) and the bottom panel illustrates the melt peaks as δ RFU over δ temperature versus temperature (°C).

Fig. 4. Post-polymerase chain reaction analysis of amplicons resulting from primer pair YES_F393 and YES_R563 in Barbara and AC Chaleur potato, as well as DNA pools of *Potato virus Y* (PVY)-resistant (R) or -susceptible (S) progeny selections from Barbara. **A**, Agarose gel electrophoresis. Lane M, 1-kb Plus DNA Ladder (Invitrogen). Sizes of the ladder DNA of 100 to 500 bp are shown on the left side of the picture. Amplicon sizes are indicated on the right of the picture. **B**, High-resolution DNA melting analysis carried out on LightScanner with respective software. The upper panel is the normalized melting curve data plotted with the normalized fluorescence against temperature (°C) and the lower panel is the normalized melting peaks with the normalized δ fluorescence versus temperature (°C). **C**, High-resolution DNA melting analysis carried out on CFX96 using the Precision Melt Analysis software. The upper panel is the melt curve data plotted as relative fluorescent units (RFU, 10^3) against temperature (°C) and the bottom panel illustrates the melt peaks as δ RFU over δ temperature versus temperature (°C).

for the markers (Supplementary Table S2). Of the 62 progeny of Barbara × G7815-9Y, 36 tested positive for both markers and 26 were negative for the markers. For progeny of Barbara × N1503-19, 30 and 34 tested positive and negative, respectively,

for both YES-HRM1 and YES-HRM2 markers. The post-PCR melting assay of a plate containing up to 96 samples on the CFX96 was performed during the PCR analysis with an additional approximately 2 min, and it took approximately 7 min to complete

Fig. 5. High-resolution DNA melting (HRM) analysis on progeny and parents of Barbara × AC Chaleur. **A**, Analysis carried out on LightScanner with respective software. Graphs were plotted as normalized melting curves (above) and normalized melting peaks (below) (X-axis) against temperature (°C) (Y-axis). The genotype visual summary of the 96-well/sample plate is shown on the left. Red indicates the resistant (R) melting profile, gray indicates the susceptible (S) melting profile, and brown indicates no-template control. **B**, Analysis carried out on CFX96 with respective software. Graphs were plotted as relative fluorescent units (RFU 10³) (X-axis) (above) and the inverse δ of RFU/ δ temperature versus temperature (°C) (Y-axis). The bottom panel is the genotype summary of the 96-well/sample plate. Red indicates the R melting profile. Polymerase chain reaction was performed using primer pair YES_F182 and YES_R345.

on the LightScanner. Phenotypic analysis of the populations produced results identical to the marker-predicted: 34:31 R/S for Barbara × Sunrise, 36:26 R/S for Barbara × G7815-9Y, and 30:34 R/S for Barbara × N1503-19 (Supplementary Figure S1). These

results, together with those of the Barbara × AC Chaleur population, demonstrate that the newly developed HRM markers are robust and accurate, and can be used for high-throughput MAS of plants carrying *Ry_{Sto}* efficiently.

Fig. 6. High-resolution DNA melting (HRM) analysis on progeny and parents of Barbara × AC Chaleur. **A**, Analysis carried out on LightScanner with respective software. Graphs were plotted as normalized melting curves (above) and normalized melting peaks (below) (X-axis) against temperature (°C) (Y-axis). The genotype visual summary of the 96-well/sample plate is shown on the left. Red and blue indicate the resistant (R) melting profiles, gray indicates the susceptible (S) melting profile, and brown indicates no-template control. **B**, Analysis carried out on CFX96 with respective software. Graphs were plotted as relative fluorescent units (RFU 10³) (X-axis) (above) and the inverse δ of RFU/ δ temperature versus temperature (°C) (Y-axis). The bottom panel is the genotype summary of the 96-well/sample plate. Red, including those circled with blue, indicates R melting profiles. Polymerase chain reaction was performed using primer pair YES_F393 and YES_R563.

DISCUSSION

MAS offers several advantages over the conventional phenotype-based selection, manifested mainly by the increased selection efficiency and precision (Collard and Mackill 2008; Ramakrishnan et al. 2015; Xu and Crouch 2008). For phenotypic selection of ER mediated by an *Ry* gene in the greenhouse, it takes approximately 3 months for a manageable number (normally approximately 200 plants, according to our experience) of progeny seedlings of a cross to be fully assessed. The process typically includes mechanical inoculation assessment of all plants and graft inoculation assessment of plants that are not infected after mechanical inoculation. To ensure that the resistance is not limited to the test strain only, further assessments with different PVY strains are also needed (Nie et al. 2015). With MAS for the same number of plants (i.e., 200 plants), however, the process can be shortened 1 to 3 weeks, depending on the DNA extraction procedure and the type of markers utilized. For markers involving PCR amplification (e.g., YES3-3A and YES3-3B for *Ry_{sto}*, RYS3C for *Ry_{adg}*, and Ry186 and Ry364 for *Ry_{che}*), the post-PCR procedure that involves gel electrophoresis for detection of the marker is the limiting factor. This is particularly evident when multiple fragments (target and nonspecific) within a close size range are amplified, as is the case for YES3-3B (Fulladolsa et al. 2015; Nie et al. in press; Song and Schwarzfischer 2008). HRM markers can significantly decrease the time and effort needed for the post-PCR analysis, requiring less than 10 min for simultaneous analysis of up to 96 samples. Evidently, in combination with high-throughput automated DNA extraction procedures (Mehle et al. 2012), HRM-based MAS for *Ry_{sto}* may only take 2 to 3 days to process approximately 200 plants, which is a significant increase in selection efficiency.

HRM analysis is widely used in human clinical laboratories as a high-throughput method for molecular diagnostics (Montgomery et al. 2010; Reed et al. 2007). HRM analysis has also been successfully applied in plants for cultivar identification (Ganopoulos et al. 2013), genotyping (Lochlainn et al. 2011), chromosome dosage assessment (Villano et al. 2015), and SNP analysis and detection (Han et al. 2011; Wu et al. 2008). HRM-based markers have also been developed in several crops for various traits or genes (Kim et al. 2016; Lehmensiek et al. 2008; Pavan et al. 2013; Terracciano et al. 2013). In potato, HRM assays for five candidate genes or markers, including the haplotype TG689 marker linked to the potato cyst nematode (PCN) *R* gene *H1*, have been developed (De Koeper et al. 2010). However, their potential for high-throughput MAS for respective traits or genes has not yet been proven, even though the assay for TG689 is being applied to selection of progeny and parents for PCN-Ro1 resistance (De Koeper et al. 2010). The development and validation of YES-HRM1 and YES-HRM2 is a further expansion of HRM technologies to high-throughput MAS for an important trait in potato, the ER to PVY.

A number of tetraploid potato cultivars, Barbara included, have been reported to carry *Ry_{sto}* and are extremely resistant to PVY infection (Barker 1996; Flis et al. 2005; Song and Schwarzfischer 2008; Song et al. 2005). In the potato breeding program of Agriculture and Agri-Food Canada (AAFC), Barbara is used as a breeding clone for development of new cultivars or selections with ER to PVY (Nie et al. in press) as well as resistance to several other economically important diseases (Stegemann and Schnick 1985). Two PVY-R selections derived from Barbara (i.e., F08086 [AR2014-09] and F08087 [AR2013-14]) have been recently released. Indeed, no ELISA-detectable level of PVY was found in Barbara after mechanical or graft inoculation with isolates belonging to PVY^O, PVY^N, PVY^{N^O}, and PVY^{NTN}, confirming its broad-spectrum ER to PVY. Interestingly, necrotic responses were observed in the newly emerging leaves after PVY-containing scions were grafted to Barbara. A similar phenomenon has been observed in plants bearing an *R* gene (e.g., *Ra* or *Ry*) in response to a corresponding virus (e.g., *Potato virus A* [PVA] or PVY) upon graft inoculation (Barker 1996; Nie and Singh 2001; Nie et al. 2015, in press). Like that proposed for

the ER in 'Shepody', which possesses *Ra*, against PVA (Nie and Singh 2001), PVY transportation or movement is not interrupted but its replication is inhibited in Barbara. When the virus titer reaches a certain level due to continuous virus supply from the scions, localized HR is triggered and, thus, the systemic spread of the virus is inhibited.

The segregation ratio of 1:1 for resistance versus susceptibility to PVY in progeny of Barbara × AC Chaleur suggests that the ER in Barbara is conferred by a simplex *Ry_{sto}* (*Rrrr*). Based on this genotype, one sequence variant that contains YES3-3A and YES3-3B is anticipated from sequences derived from the chromosome region (chr12) harboring these markers. Indeed, only one sequence variant (*V_R*) in every four sequences in Barbara and the pool of PVY-R selections (F00069, F08086, and F08087) derived from Barbara contained the markers. Among the nonmarker-bearing sequences, one variant (*V_{r1}*) accounted for one-fourth the sequences, whereas the other (*V_{r2}*) accounted for two-fourths, leading to an overall ratio of 1:1:2 (*V_R*/*V_{r1}*/*V_{r2}*), with a genotype of *Rr₁r₂r₂*. In AC Chaleur and the pool of PVY-S selections (F12080 and F12081), no marker-containing variants were found, and the two sequence variants (*V_{r1}* and *V_{r2}*) accounted for 50% each, with a precise genotype of *r₁r₁r₂r₂*. The nucleotide variations or polymorphisms, which lead to different melting profiles, are reflected in more than one melting peak in a given sample (Figs. 3 to 6). It is interesting to note that some progeny deemed PVY-R did not possess the small melting peak at approximately 72.5°C in the YES-HRM2 assay; instead, they exhibited a distinct peak at approximately 80°C (Fig. 6). The altered melting profile in some R progeny might be attributed to the interactions between the amplicon from *V_R* and those from *V_{r1}* or *V_{r2}*. For the YES-HRM1 assay, the 20-nt deletion in *V_R* lowered the melting temperature by approximately 2°C, thus enabling an effective detection of the YES-HRM1 marker in PVY-R progeny and parent.

The complete agreement between the YES3 markers and phenotypes in four segregating populations involving Barbara as the R parent demonstrates that YES3 markers are cosegregating with *Ry_{sto}* from Barbara. The YES3-3A and YES3-3B markers have successfully identified ER to PVY in 188 European cultivars (Kuhl 2011); although not yet tested, it is expected that the newly developed YES-HRM1 and YES-HRM2 should give identical results as the STS markers for the identification of ER in these cultivars.

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