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ORIGINAL PAPER

Development of allele-specific PCR and RT–PCR assays for clustered resistance genes using a potato late blight resistance transgene as a model

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Abstract Members of the NBS-LRR gene family impart resistance to a wide variety of pathogens and are often found clustered within a plant genome. This clustering of homologous sequences can complicate PCR-based characterizations, especially the study of transgenes. We have developed allele-specific PCR and RT–PCR assays for the potato late blight resistance gene *RB*. Our assay utilizes two approaches toward primer design, allowing discrimination between the *RB* transgene and both the endogenous *RB* gene and numerous *RB* homeologs. First, a reverse primer was designed to take advantage of an indel present in the *RB* transgene but absent in *rb* susceptibility alleles, enhancing specificity for the transgene, though not fully discriminating against *RB* homeologs. Second, a forward primer was designed according to the principles of mismatch amplification mutation assay (MAMA) PCR, targeting SNPs introduced during the cloning of *RB*. Together, the indel reverse primer and the MAMA forward primer provide an assay that is highly specific for the *RB* transgene, being capable of distinguishing the transgene from all *RB* endogenous gene copies and from all *RB* paralogs in a diverse collection of wild and cultivated potato genotypes. These primers have been successfully multiplexed with primers of an internal control. The multiplexed assay is useful for both PCR and RT–PCR applications. Double MAMA-PCR, in which both PCR primers target separate transgene-specific SNPs, was also tested and shown to be equally specific for the *RB* transgene. We propose extending the use of MAMA for the characterization of resistance transgenes.

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Introduction

Plant resistance against pathogens has received substantial research attention in recent years, resulting in noteworthy advances in the study and cloning of resistance (R) genes. Dozens of R genes imparting resistance against about as many pathogens have been cloned and characterized to date. The vast majority of cloned R genes are members of the nucleotide binding site-leucine rich repeat (NBS-LRR) gene class. NBS-LRR gene families may be of ancient origin and representatives are found in both monocots and dicots. Presumably, particular pathogen specificities arose within NBS-LRR gene families through a process of coevolution with pathogens (Vleeshouwers et al. 2001). A strong characteristic of these genes is that they are found clustered throughout both monocot and dicot genomes (Leister et al. 1996; Pan et al. 2000b).

Analysis of the complete genome sequence of *Arabidopsis thaliana* has allowed the determination of total numbers of NBS-LRR resistance genes in the species. *Arabidopsis* has 43 NBS-LRR clusters (Meyers et al. 2003), averaging 4.9 sequences per cluster, although clusters comprised of as many as 40 sequences have been identified (Meyers et al. 1999).

Each cluster is not necessarily dedicated to a particular pathogen, as genes within a cluster may or may not have diverse pathogen specificities (Leister et al. 1996; Michelmore and Meyers 1998; Rossi et al. 1998; Richter and Ronald 2000; van der Vossen et al. 2000). Similarly, related plants may have syntenic regions of clustered R genes, though pathogen specificity is not necessarily conserved (Leister et al. 1996; Grube et al. 2000).

Ever since the European Potato Famine in the mid-1800s, there has been a concerted effort to control the oomycete *Phytophthora infestans*, causal agent of late blight. Yield losses in developed nations due to this disease can reach 15% and late blight continues to result in multi-billion dollar losses worldwide each year (Kamoun 2001; Garelik 2002). A large portion of potato production costs is attributable to preventative measures, especially fungicide use. Because the predominant US *P. infestans* genotype, US8, is resistant to the systemic fungicide metalaxyl (Fry and Goodwin 1997), the disease cannot be stopped once it starts. To prevent late blight losses, fungicides may be used even when growing conditions are not favorable for disease development. In an effort to reduce production costs, there has been a search for genetic resistance to late blight.

As with other plant-microbe interactions, substantial research effort has focused on cloning R genes for late blight resistance (Ballvora et al. 2002; Song et al. 2003; Huang et al. 2005; Park et al. 2005; van der Vossen et al. 2003, 2005). Of particular potential for potato improvement is *RB* (also known as *Rpi-blb1)*, cloned from the wild potato species *S. bulbocastanum* (Song et al. 2003; van der Vossen et al. 2003). *RB* is a member of the large NBS-LRR gene class, and, like most other NBS-LRR genes, occurs in a cluster of related sequences (79.6–85.6% similarity). Besides *RB*, three seemingly intact paralogs of no known function and an apparent pseudogene have been described (Song et al. 2003). At least two additional genome locations contain *RB*-related sequences as well (Pan et al. 2000a; van der Vossen et al. 2003). Two *RB* alleles, a "resistance" allele and a "susceptibility" allele, have been described (Song et al. 2003). These are 99.8% similar with an 18 bp indel present in the resistance allele. An *RB* transgene, under the control of the native promoter, was generated from genomic DNA via long range (LR)–PCR, which resulted in the introduction of three SNPs in the coding region (Song et al. 2003). These SNPs have no obvious effect on transgene function. Cultivated potatoes 'Katahdin,' 'Russet Burbank,' 'Dark Red Norland,' and 'Superior' transformed with *RB* show strong foliar resistance characterized by slowed lesion development and suppression but not elimination of sporulation (Song et al. 2003; Lozoya-Saldana et al. 2005; and unpublished). Even in the Toluca Valley of Mexico, genotypic center of diversity for *P. infestans*, the *RB* transgene is effective, indicating *RB* lacks pathogen race specificity (Song et al. 2003; Lozoya-Saldana et al. 2005).

Hypotheses on the cellular role and regulation of NBS-LRR genes have emerged. The products of NBS-LRR genes are similar to intracellular receptors (Richter and Ronald 2000) and act as sentinels involved in pathogen detection and defense response initiation (Nimchuk et al. 2001). In their sentinel role, R proteins need to be present in the cell prior to pathogen attack. Accordingly, NBS-LRR genes are presumed to be constitutively transcribed throughout plant development, even in the absence of the pathogen. In contrast, there is a rich literature documenting the developmental regulation of foliar late blight resistance in potato at the phenotypic level. Resistance increases during early stages of plant development, peaking at the time of flowering, and then decreasing as the plant ages and begins to senesce (Carnegie and Colhoun 1982; Stewart et al. 1983; Stewart 1990). Now that R genes for potato late blight control have been cloned (Ballvora et al. 2002; Song et al. 2003; Huang et al. 2005; Park et al. 2005; van der Vossen et al. 2003, 2005), it is possible to explore the effects of varying plant developmental age on specific R gene function and transcription. Understanding this phenomenon is essential for successful *RB* deployment and will expand our understanding of how NBS-LRR genes in general are regulated.

Towards understanding *RB* transcriptional regulation, PCR and RT–PCR assays specific for the *RB* transgene and functional in a wide array of potato genotypes are needed. The existence of both clustered *RB* paralogues and *RB* homologs in other genome locations present challenges for the design of transgene specific assays for this locus. SNPs introduced into the *RB* transgene via LR–PCR provide convenient transgene-specific targets for primer design. Mismatch amplification mutation assay (MAMA)–PCR capitalizes upon SNP differences between alleles to yield highly specific assays (Cha et al. 1992). In MAMA–PCR, primers are designed such that the 3′ ultimate nucleotide encompasses a SNP differentiating target and non-target sequences. Additionally, MAMA primers contain a deliberately introduced nucleotide mismatch at the penultimate position. As a result, when the primer anneals to non-target sequences, the last two bases of the primer do not anneal, preventing primer extension. In target alleles, there is only one

mismatch, the penultimate base, allowing the last base to anneal and primer extension to occur. MAMA–PCR has primarily been used in animal systems for pathogen and cancer studies (Wagner et al. 1997; Zirnstein et al. 2000; Jinneman and Hill 2001; Qiang et al. 2002). Few MAMA–PCR studies have been performed in plants although Drenkard et al. (2000) used MAMA–PCR at the genome level to create molecular markers for map-based cloning in *Arabidopsis*. PCR markers linked to genes conditioning rice blast resistance have been created using an allele-specific, MAMA–PCR-like approach in which a discriminating SNP is positioned at the 3′ ultimate nucleotide of the primer and an additional mismatch is introduced within the last four nucleotides (Hayashi et al. 2004, 2006). To our knowledge, MAMA–PCR has not been previously used to study resistance gene transcription nor differences between transgenes and endogenous genes.

Like most plant R genes, *RB* is a member of the NBS-LRR class and exists within a cluster of related sequences. As additional R alleles are characterized in various plant species and as additional R transgenes are generated via LR–PCR (Sanchez and Bradeen 2006), approaches to differentiate between highly similar R alleles are needed. Here we describe the development of MAMA–PCR and RT–PCR assays specific for the *RB* transgene. The assays are so sensitive they can differentiate between the *RB* transgene and the endogenous gene copy from which it was cloned. The assays function successfully in a range of genetic backgrounds and may serve as a model for the development of similar allele-specific assays in other R gene systems.

Materials and methods

Plant material

Solanum bulbocastanum genotype PT29, cultivated potato [*S. tuberosum* cultivars Katahdin (lines SP922, SP951, SP966) and Russet Burbank (lines SP2105, SP2211, SP2212, SP2213)] carrying the *RB* transgene, and untransformed cultivated potato (*S. tuberosum* cultivars Katahdin and Russet Burbank) were kind gifts from John Helgeson (USDA-ARS and University of Wisconsin, Madison) and Jiming Jiang and Sandra Austin-Phillips (University of Wisconsin, Madison). Potato cultivars Shepody, AC Blue Pride, AC Brador, Arran Pilot, Bintje, Fortyfold, French Fingerling, Corne de Montan, Alturas, US W842 were obtained from Bill Campbell (Alaska Department of Natural Resources) or Shelley Jansky (USDA-ARS and University of Wisconsin, Madison). Additional *S. bulbocastanum* genotypes (PI 243345, 243509, 243510, and 275188) were grown from seed originating from the USDA Potato Genebank (Sturgeon Bay, WI). All plant materials were produced in greenhouses on the University of Minnesota (St. Paul, MN) campus or at the Sand Plain Research Farm (Becker, MN) using standard cultural practices. For molecular assays, the third leaf below the crown of the plant was collected, frozen immediately in liquid nitrogen, and stored at −80°C.

Primer design

All primers used in this study were developed from *RB* transgene sequence (GenBank number 32470640) using the Primer3 software (http://www.frodo.wi.mit.edu) or were previously published [5′RACE (previously listed as "2–5′-RACE"), Song et al. 2003]. Primer sequences are listed in Table 1. Relative primer locations are depicted in Fig. 1. All primers were obtained from Integrated DNA Technologies (Coralville, IA).

For MAMA–PCRs and RT–PCRs, MAMA primers were designed to take advantage of *RB* transgene-specific SNPs introduced by LR–PCR (Song et al. 2003). Primers were developed such that the transgene-specific SNP is located at either the ultimate or penultimate 3′ position of the primer. When the SNP was placed at the 3′ end of the primer, the penultimate base was intentionally changed to one of the three possible mismatch bases (e.g., for MAMA1–MAMA3, the penultimate A is changed to G, C, and T, respectively; Table 1). When the transgene-specific SNP was penultimate, the last base was intentionally changed to one of the three possible mismatch bases (e.g., for MAMA4–MAMA6, the last base is changed from G to A, C, and T, respectively; Table 1). MAMA primers were designed to be 24–27 bases in length with a calculated (Primer3) melting temperature of 58–60°C.

Non-MAMA (standard) primers include an indel reverse primer, INDEL-r, designed to take advantage of an 18 bp indel present in the *RB* transgene and the endogenous *S. bulbocastanum RB* allele, but absent in the endogenous *S. bulbocastanum rb* (susceptibility) allele and previously characterized *S. bulbocastanum RB* homologs (Fig. 1; Table 1). NONMAMA and RB-f are standard forward primers. NONMAMA incorporates the transgene-specific SNP as the ultimate base, with no mismatch at the penultimate base position. The 3′ most nucleotide (corresponding to the transgene-specific SNP) was removed from NONMAMA to generate primer RB-f. As an internal control for *RB* transgene PCRs and RT–PCRs, primers RP2-f1 and RP2-r5 (collectively referred to as "RP2 primers")

Table 1 PCR, RT–PCR, and sequencing primers

Forward primers	Sequence[a]	Tm[b]
RB-f	CATCTTGAGAGAGTGAAGAATGATA	56.86
NONMAMA	CATCTTGAGAGAGTGAAGAATGATA*T*	57.26
MAMA1	CATCTTGAGAGAGTGAAGAATGATg*T*	60.19
MAMA2	CATCTTGAGAGAGTGAAGAATGATc*T*	59.47
MAMA3	CATCTTGAGAGAGTGAAGAATGATtT	59.36
MAMA4	CATCTTGAGAGAGTGAAGAATGATA*T*a	57.36
MAMA5	CATCTTGAGAGAGTGAAGAATGATA*T*c	58.91
MAMA6	CATCTTGAGAGAGTGAAGAATGATA*T*t	58.50
2MAMA5′1	TTGCAACCATATGAACTGTCAAg*C*	63.40
2MAMA5′2	TTGCAACCATATGAACTGTCAAt*C*	61.12
2MAMA5′3	TTGCAACCATATGAACTGTCAAc*C*	63.17
RP2-f1	TCGTGGATTTTTCCGATCTC	60.01
M13 (−20)	GTAAAACGACGGCCAGT	53.0
Reverse primers	**Sequence**	
5′RACEc	ATCCACTTCTTCAACATACTCCACATCC	65.53
INDEL-r	CTAGTGCGCAACACAATTGAA	59.93
2MAMA3′1	CCAGAGAGAGATTAGCTTTTCTTTTc*T*	60.39
2MAMA3′2	CCAGAGAGAGATTAGCTTTTCTTTTa*T*	58.46
2MAMA3′3	CCAGAGAGAGATTAGCTTTTCTTTTg*T*	61.02
RP2-r5	ATCTCGCTCCATCTCTCCAA	59.91
M13 (−27)	CAGGAAACAGCTATGAC	47.3

[a] Listed 5′–3′. LR–PCR introduced SNPs in italics; MAMA mismatches—lowercase

[b] Melting temperature in °C, calculated by Primer3

[c] 2–5′-RACE, Song et al. 2003

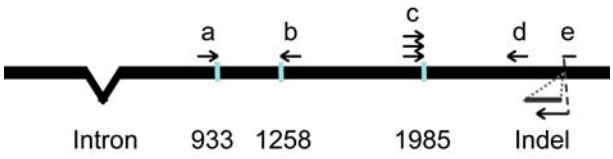

Fig. 1 *RB* transgene structure. *RB* (resistance allele) indel and LR–PCR introduced SNP locations (933, 1258, 1985) are indicated. Primer locations and directions are indicated: **a** 2MAMA5′x; **b** 2MAMA3′x; **c** RB-f, NONMAMA, MAMAx; **d** 5′RACE; **e** INDEL-r (see Table 1). Drawing not to scale

were developed for *RNA Polymerase II* subunit 2, based on sequence from tomato, *S. esculentum* (GenBank number 1049067).

PCR and RT–PCR methods

DNA and total RNA were extracted from frozen leaf tissue. For DNA extractions, a modified cTAB protocol (Fulton et al. 1995) was used. Total RNA was extracted using the SV Total RNA Isolation kit (Promega, Madison, WI) according to manufacturer's recommendations.

PCRs were performed using AmpliTaq™ (Applied Biosystems, Foster City, CA) in a 50 µl total volume [15 ng template, 1 µM each primer, 200 µM each dNTP, 1.25 units Taq, 10× buffer (provided by manufacturer)]. Three-step PCRs used 35 cycles of 94°C 15″, 57°C 30″, 68°C 50″. To test amplification conditions, the three-step reaction was modified by altering the annealing temperature in two degree increments, ranging from 51 to 61°C. Two-step PCRs utilized a combined primer anneal/extension step with 35 cycles of 94°C 15″, 61°C 1′ 20″. Annealing temperature was also varied in the two step reactions in two degree increments, ranging between 53 and 63°C, to test amplification conditions. Invitrogen's SuperScript™ one-step RT–PCR kit (Carlsbad, CA) was used for RT–PCRs, performed in a 50 µl total volume according to manufacturer's recommendations. For multiplexed RT–PCRs, RP2 primers were tested at 1, 0.5, 0.25, and 0.1 µM concentrations while *RB* transgene primers were maintained at a 1 µM concentration. After an initial reverse transcription cycle of 30 min at 50°C, RT–PCRs underwent 35 cycles of 94°C 15″, 57°C 30″, 68°C 50″. PCR and RT–PCR products were separated on 1% agarose gels in TBE buffer, stained with ethidium bromide, and photographed under UV light.

All nucleotide sequences were determined by Applied Biosystems BigDye Terminator cycle sequencing on an Applied Biosystems 3100 or 3700 automatic sequencer at the University of Minnesota Advanced Genetic Analysis Center. PCR products were either desalted through a MicroSpin™ S-300 HR column (Amersham Biosciences, Piscataway, NJ) according to manufacturer's recommendations and sequenced directly using 3.2 ρmole of primer MAMA2 or INDEL-r, or were cloned into the pGEM®-T Easy Vector (Promega) according to manufacturer's instructions. Plasmids were purified from 3 ml overnight

cultures using the Wizard *Plus* SV Minipreps DNA Purification system (Promega) following manufacturer's recommendations. To verify insert size, 3 µl of plasmid DNA were digested in 1× manufacturer supplied buffer by 10 units *Eco*RI (Invitrogen) in a 10 µl total volume at 37°C for 1 h. The entire reaction was loaded and separated on 1% agarose gels in TBE buffer, stained with ethidium bromide, and photographed under UV light. Inserts were compared to DNA standards of known size. Subsequently, undigested plasmids were sequenced using 3.2 pmole of M13 forward or reverse primers. Sequences were analyzed and assembled into contigs using SeqMan™ II (Windows 32 vs. 5.08; DNASTAR Inc, Madison, WI). Homology of the contigs to known sequences was determined using BLASTn (NCBI, http://www.ncbi.nlm.nih.gov/blast/).

Results

Primer specificity required

RB is a member of a cluster of five related sequences (Song et al. 2003; van der Vossen et al. 2003). Only the *RB* transgene was introduced into cultivated potato, but endogenous homeologous sequences are found in at least three genome locations (Pan et al. 2000a; van der Vossen et al. 2003). Primers were designed from the *RB* coding region (RB-f and 5′ RACE; Table 1, Fig. 1). These primers do not target transgene-specific SNPs and should amplify *RB* alleles, homologs, and homeologs. Accordingly, fragments of the expected target size (363 bp) were amplified in all tested samples of wild and cultivated (untransformed and transformed) potato (Fig. 2a). To increase primer specificity for the *RB* transgene, a primer encompassing an indel present in the *RB* transgene and the endogenous *S. bulbocastanum RB* (resistance allele), but absent in other previously characterized *RB* alleles and homologs, was designed (INDEL-r, Table 1). Primer INDEL-r was paired with RB-f, but again, *RB* homolog and homeolog fragments of the target size (363 bp) were detected in all samples tested (Fig. 2d). While the 18 bp indel present in characterized *RB* (resistance) alleles and absent in *rb* (susceptibility) alleles provides opportunity to develop primers that differentiate between alleles characterized to date, the indel is apparently present in *S. bulbocastanum RB* homologs and in *S. tuberosum RB* alleles and/or homeologs. Thus, a primer based on the *RB* transgene indel does not alone impart the desired specificity.

We next targeted the design of an improved forward primer for increased transgene specificity. The *RB* transgene was generated via LR–PCR, resulting in the introduction of three SNPs in the coding region relative to the endogenous gene (Song et al. 2003). These SNPs are located in the coding region at nucleotide positions 933 (T→C), 1258 (G→A), and 1985 (A→T) (Fig. 1). Only SNP 933 resulted in a synonymous residue change. These SNPs have no obvious effect on transprotein function, but can be utilized to design primers specific to the transgene. A forward primer, NONMAMA, was designed by placing SNP 1985 at the ultimate 3′ position of the primer (Table 1). Forward primer NONMAMA was paired with reverse primers 5′RACE (Fig. 2b) and INDEL-r (Fig. 2e) in separate reactions. Yet again, all transgenic and non-transgenic wild and cultivated potato samples tested yielded appropriately sized fragments (363 bp). Although forward primer NONMAMA targeted an *RB* transgene-specific SNP at its extreme 3′ position, selective amplification of the transgene was not achieved (Fig. 2b, e).

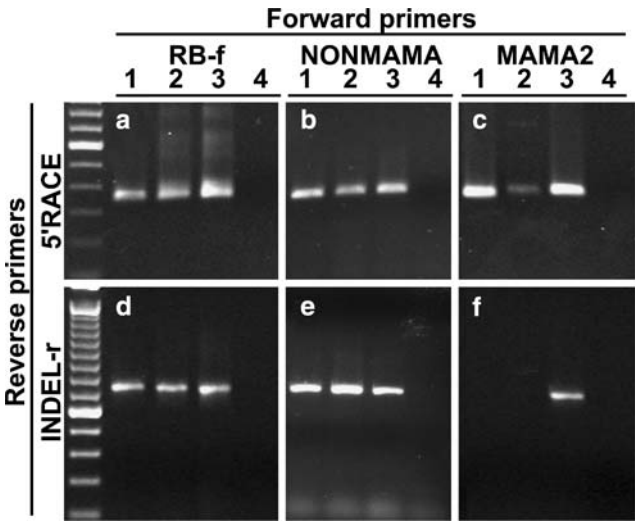

Fig. 2 The indel reverse primer and MAMA forward primer are specific for the *RB* transgene. PCRs using wild and cultivated [non-transgenic and transgenic (+*RB*)] potato genotype DNA templates (*1 S. bulbocastanum* PT29; *2 S. tuberosum* cultivar Katahdin; *3 S. tuberosum* cultivar Katahdin + *RB* SP922; *4* H_2O control) and forward and reverse primers as indicated. **a**, **d** RB-f lacks both LR–PCR introduced and MAMA mismatches, and is therefore not specific to the transgene nor to the endogenous *RB* when paired with either the non-*RB* transgene specific 5′RACE or the indel-based INDEL-r. RB-f does not provide specificity for the *RB* transgene. **b**, **e** NONMAMA terminates in a LR–PCR generated, transgene-specific SNP, but is not sufficient to impart specificity for the transgene when paired with 5′RACE or INDEL-r. **c** MAMA2 when paired with 5′RACE does not have sufficient specificity for the *RB* transgene. **f** However, primer pair MAMA2–INDEL-r is highly specific for the *RB* transgene, differentiating between the endogenous *S. bulbocastanum RB* gene, *S. bulbocastanum RB* homologs, and cultivated potato *RB* homeologs. Expected fragment sizes: 363 bp (**a**, **b**, **c**), 730 bp (**d**, **e**, **f**)

MAMA

We next tested MAMA forward primers incorporating the *RB* transgene-specific SNP 1985. Two types of MAMA primers were tested: (1) primers that incorporated the *RB* transgene-specific SNP at the extreme 3′ nucleotide position of the primer and an intentional mismatch at the penultimate nucleotide position (MAMA1, MAMA2, and MAMA3) and (2) primers that incorporate the *RB* transgene-specific SNP at the penultimate nucleotide position and an intentional mismatch at the extreme 3′ nucleotide position (MAMA4, MAMA5, and MAMA6). For both types of primers, every possible nucleotide mismatch was considered. That is, if the targeted mismatch base was originally an A, separate MAMA primers incorporating T, C, and G were analyzed. In total, six forward primers were tested (Table 1).

MAMA primers alone are not sufficient for specific amplification of the *RB* transgene, as shown when paired with non-*RB* specific reverse primer 5′RACE (Fig. 2c). The added specificity provided by the reverse primer INDEL-r is also required for specific transgene detection (Fig. 2f). Each of the MAMA forward primers was paired with INDEL-r (Fig. 1). In general, MAMA primers with mismatches in the penultimate position (MAMA1–3) yielded greater specificity, with MAMA3–INDEL-r yielding robust amplification of the *RB* transgene but no amplification of the endogenous *RB* gene copy, *S. bulbocastanum RB* homologs, or cultivated potato *RB* homeologs (Fig. S1).

Double MAMA

MAMA primers paired with a reverse primer lacking transgene specificity failed to yield an assay specific for the *RB* transgene (Fig. 2c), possibly due to the extensive representation of *RB*-like sequences in the potato and *S. bulbocastanum* genomes. In contrast, primer pair MAMA2–INDEL-r yielded a specific assay for the *RB* transgene. However, allele-specific indels are not a feature of every R allele or R transgene system. To demonstrate that a MAMA approach can be adapted for allele-specific assays in other R gene systems, we developed a double MAMA (2MAMA) approach in which both forward and reverse primers contain a penultimate mismatch and incorporate a 3′ SNP characteristic of the target allele.

For our double MAMA assays, SNPs located at positions 933 and 1258 of the *RB* transgene were targeted for primer design (Fig. 1; Table 1). SNP 933 was used for design of forward primers (2MAMA5′1, 2MAMA5′2, and 2MAMA5′3) and SNP 1258 was used for design of reverse primers (2MAMA3′1, 2MAMA3′2, and 2MAMA3′3). Each 2MAMA forward primer was paired with each 2MAMA reverse primer in separate PCRs (Fig. 3). Forward primers 2MAMA5′1 and 2MAMA5′3 and reverse primers 2MAMA3′1 and 2MAMA3′2 effectively amplified the *RB* transgene. The best combination in terms of amplification specificity and robustness was 2MAMA5′3 and 2MAMA3′1. In all combinations, primers 2MAMA5′2 and 2MAMA3′3 yielded no or only faint *RB* transgene amplicons (Fig. 3). Our results indicate that a double MAMA approach to primer design can yield target specificity comparable to the MAMA-indel approach detailed above. However, for the *RB* transgene, we opted to further optimize the MAMA-indel assay.

PCR settings are critical to specificity

The specificity of MAMA primers results from imperfect annealing to genomic DNA template during the first PCR cycle. Therefore, optimized PCR settings, and especially optimized annealing temperatures, are critical to obtain specificity for the *RB* transgene. We explored the effects of varying annealing temperatures on specificity of MAMA primers incorporating a penultimate mismatch (MAMA1–3; Fig. 4, S2). Our results indicate that the level of detection of *RB* homeologs can be greatly decreased, if not eliminated, by varying the annealing temperature. Non-target amplification of fragments larger and smaller than the expected size occurs when annealing temperatures are low (53 or 55°C). Higher temperature reactions (57, 59, or 61°C) yielded only the target amplicon, although band robustness can be compromised as annealing temperature increases (Fig. 4). For the primers tested, the optimal annealing temperature in terms of amplification specificity and robustness is 57°C. At that temperature, all three MAMA primers incorporating the *RB* transgene-specific SNP at the penultimate position (MAMA1–3), when paired with the INDEL-r primer, amplify the *RB* transgene to the exclusion of *RB* homologs and homeologs (Fig. 4). However, even under less than optimal PCR conditions, the primer pair MAMA2 and INDEL-r preferentially amplifies the *RB* transgene over the endogenous gene and its homologs (Fig. S1).

Cha et al. (1992) combined the primer annealing and amplicon extension steps, yielding a two-step PCR, when characterizing the rat c-Ha-*ras* gene. We tested the effectiveness of a two-step PCR, pairing forward primers MAMA1–3 and reverse primer INDEL-r. Non-target amplification at all but one tested temperature (61°C) suggested that two-step PCR might not

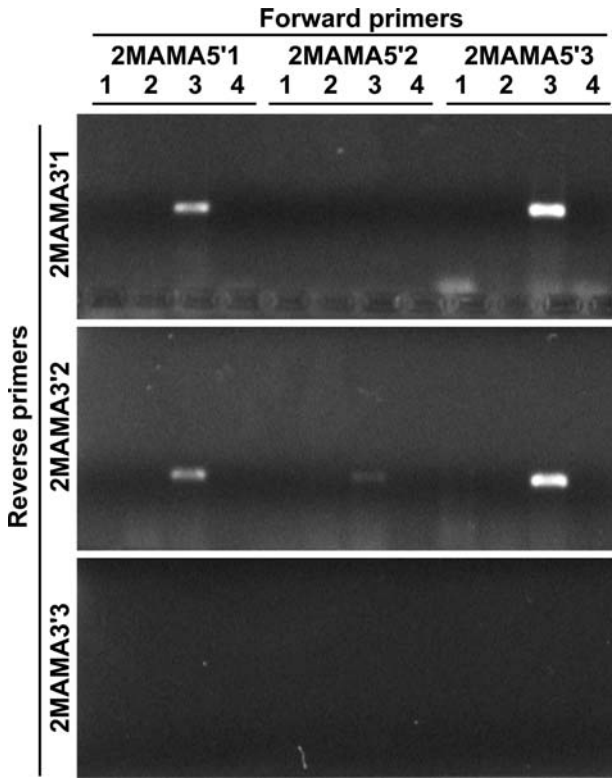

Fig. 3 Double MAMA-PCR selectively amplifies the transgene *RB*. Templates include genomic DNA from plants with endogenous *RB* and *RB* homologs (*1 S. bulbocastanum* PT29), untransformed cultivated potato with *RB* homeologs (*2 S. tuberosum* cultivar Katahdin), transformed cultivated potato with *RB* homeologs and the *RB* transgene (*3 S. tuberosum* cultivar Katahdin + *RB* SP922), and a negative control (*4* H_2O). All reactions utilized an annealing temperature of 57°C. Expected fragment size: 375 bp. For double MAMA, forward primers (2MAMA5′x) were paired with reverse primers (2MAMA3′x) to assess the specificity of each of the nine possible primer pairs. Primer sequences are listed in Table 1. Primers 2MAMA3′3 and 2MAMA5′2 function poorly, with little or no target amplicon in reactions utilizing these primers. Under the conditions tested, 2MAMA5′3 and 2MAMA3′1 yielded specific and robust amplification of the *RB* transgene to the exclusion of the *S. bulbocastanum* endogenous *RB* gene and *RB* homologs and of the cultivated potato *RB* homeologs. Both 2MAMA5′3 and 2MAMA3′1 incorporate a C as the introduced penultimate mismatch

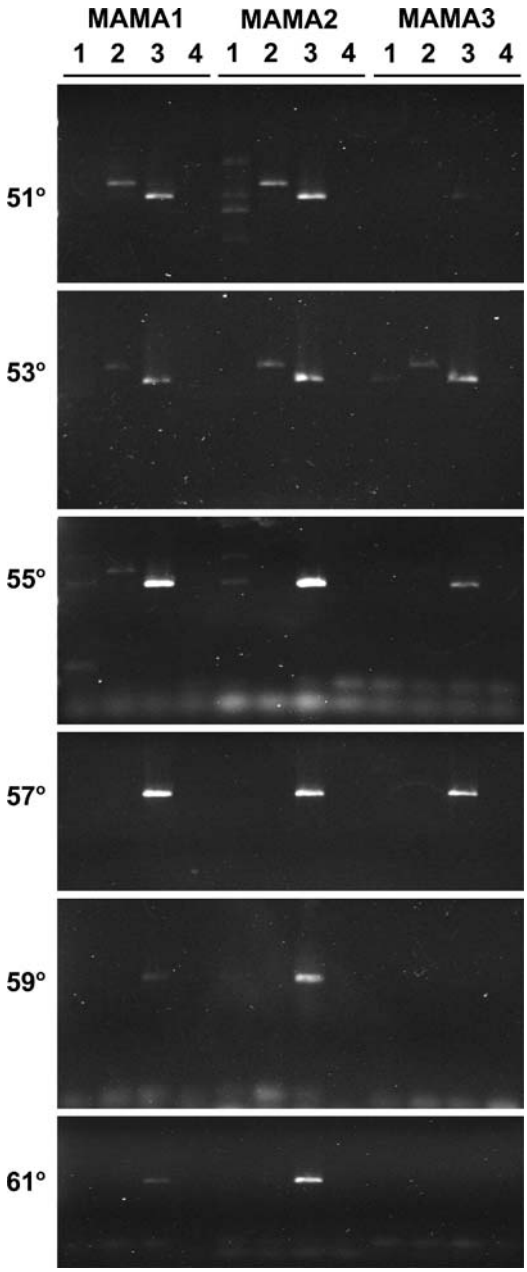

Fig. 4 Thermocycler conditions, especially annealing temperature, affect MAMA–PCR specificity (Three-step PCR). Results of PCRs using MAMA forward primers and the INDEL-r reverse primer. Templates include genomic DNA from plants with endogenous *RB* and *RB* homologs (*1 S. bulbocastanum* PT29), untransformed cultivated potato with *RB* homeologs (*2 S. tuberosum* cultivar Katahdin), transformed cultivated potato with *RB* homeologs and the *RB* transgene (*3 S. tuberosum* cultivar Katahdin + *RB* SP922), and a negative control (*4* H_2O). Expected fragment size: 730 bp. Non-specific amplification occurs at lower annealing temperatures. Specificity occurs at higher annealing temperatures, although efficiency is compromised at the upper extreme tested

consistently yield target amplification (Fig. S2). We have therefore chosen to use the traditional three-step PCR with an annealing temperature of 57°C for subsequent *RB* analysis.

RB transgene PCR and RT–PCR assays

The *RB* transgene-specific fragment generated by primer pair MAMA2–INDEL-r is a dominant marker assay, with no *RB* transgene amplicon present in untransformed potato. To differentiate between a true *RB* transgene negative reaction and a failed PCR, we multiplexed primer pair MAMA2–INDEL-r with primers designed from *RNA Polymerase II* (*RP2*). *RP2* is highly conserved across taxa and is self-regulating,

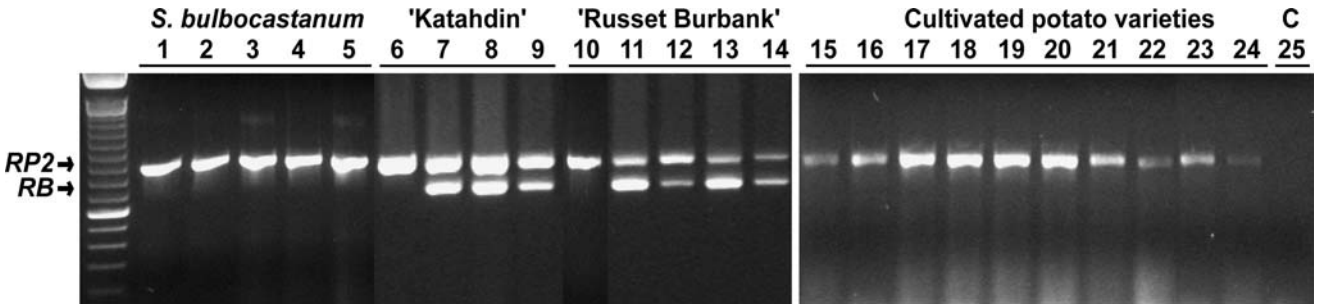

Fig. 5 Multiplex PCR using *RB* transgene-specific primers and *RNA Polymerase II* primers. MAMA2 and INDEL-r primers in combination with internal control primers RP2 developed from the *RNA Polymerase II* gene were used to amplify fragments from genomic DNA of wild and cultivated (non-transgenic and transgenic) potato genotypes (*1 S. bulbocastanum* PT29; *2 S. bulbocastanum* PI 243345; *3 S. bulbocastanum* PI 243509; *4 S. bulbocastanum* PI 243510; *5 S. bulbocastanum* PI 275188; *6* 'Katahdin' untransformed; *7* 'Katahdin' + *RB* SP922; *8* 'Katahdin' + *RB* SP951; *9* 'Katahdin' + *RB* SP966; *10* 'Russet Burbank' untransformed; *11* 'Russet Burbank' + *RB* SP2211; *12* 'Russet Burbank' + *RB* SP2212; *13* 'Russet Burbank' + *RB* SP2213; *14* 'Russet Burbank' + *RB* SP2105; *15* 'Shepody'; *16* 'AC Blue Pride'; *17* 'AC Brador'; *18* 'Arran Pilot'; *19* 'Bintje'; *20* 'Forty-fold'; *21* 'French Fingerling'; *22* 'Corne de Montan'; *23* 'Alturas'; *24* 'US W842'; *25* H_2O control). RP2 is amplified in all samples (expected fragment size: 862 bp), indicating this gene is present in all samples and the PCRs were successful. The *RB*-transgene amplicon (expected fragment size: 730 bp) is present only in those samples that contain the *RB* transgene (lanes 7–9, 11–14)

suggesting that mRNA levels will not greatly fluctuate within an organism or over time. Consistently, in humans it was found that *RP2* RNA transcription is constant across tissues and treatments (Radonić et al. 2004). Thus, *RP2* is a useful positive control for both PCR and RT–PCR assays. In multiplexed reactions, smaller PCR fragments are amplified with greater efficiency (Nie and Singh 2001). Therefore, we designed *RP2* primers to yield an 862 bp fragment, larger than the 730 bp *RB* transgene-specific fragment generated by primer pair MAMA2–INDEL-r.

A survey of multiple *S. bulbocastanum* and cultivated potato genotypes was performed to determine the specificity of the MAMA2–INDEL-r primer set. Genotypes were chosen from the central Mexican range of *S. bulbocastanum*. Included in this survey was *S. bulbocastanum* genotype PT29, the genotype from which the *RB* transgene was generated. Diverse heirloom and popular varieties of cultivated potato were also selected. Since each of these genotypes lacks the *RB* transgene, we would expect no *RB* transgene amplicon. In the same survey, we included three independently transformed lines of *S. tuberosum* cultivar Katahdin and four independently transformed lines of cultivar Russet Burbank. DNA from each genotype served as template for multiplexed PCR. Although each genotype surveyed yielded the 862 bp *RP2* fragment (verified by sequence analysis), indicating successful PCR conditions, the 730 bp *RB* transgene fragment (verified by sequence analysis) was amplified only in transgenic samples (Fig. 5). Significantly, the *RB* transgene-specific fragment was missing in *S. bulbocastanum* genotype PT29, confirming that primer pair MAMA2–INDEL-r is so highly specific for the *RB* transgene as to differentiate the *RB* transgene from the endogenous allele from which it was cloned.

We next tested the utility of our MAMA2–INDEL-r primer set for RT–PCRs on *S. bulbocastanum* and cultivated potato (untransformed and transformed 'Katahdin' and 'Russet Burbank', Fig. 6). Total RNA served as template for all reactions. Robust amplification of the *RB*-transgene product (expected fragment size: 730 bp) was observed in transgene-carrying samples when the MAMA2–INDEL-r primer set was used alone (Fig. 6a). No amplification was observed in the untransformed cultivated potato and *S. bulbocastanum* samples, consistent with the PCRs performed previously on the same genotypes (Fig. 5). Next, a multiplexed RT–PCR of the MAMA2–INDEL-r and RP2 primer sets was performed using the same total RNA templates (Fig. 6b). It was observed that *RP2* primer concentration dramatically affected the ability to detect the *RB* transgene product (not shown). Although the *RP2* product is larger than the *RB* transgene product and smaller products are normally preferentially amplified, the use of 1 μM each of *RP2* forward and reverse primers limited *RB* transgene amplification. In contrast, this primer concentration worked well for PCRs (Fig. 5), suggesting that differential amplification in RT–PCRs may be due to more robust transcription of *RP2* than the *RB* transgene. RP2 primer concentrations were subsequently tested at 1, 0.5, 0.25, and 0.1 μM concentrations (not shown). The concentration of 0.25 μM for each *RP2* primer and 1 μM for each *RB* transgene primer provided the best compromise between detection of the *RP2* and *RB* transgene

amplicons, allowing detection of *RP2* transcript in all samples but the *RB* transgene transcript only in the transgenic cultivated potato samples (Fig. 6b). Additionally, in multiplexed RT–PCRs but not in multiplexed PCRs, an amplicon of 489 bp was detected in all wild and cultivated potatoes surveyed. Sequence analysis revealed that this amplicon has homology to late blight resistance genes previously identified in cultivated potato [SH20 (95% identity, GenBank number 39636799) and SH10 (93% identity, GenBank number39636784)] and the wild potato *S. tarijense* [T118 (94% identity, GenBank number 39636815)]. This fragment also shares 88% identity with the *RB* transgene. The 489 bp amplicon was generated by primers MAMA2 and RP2-r5. As expected, the strength of this band appears to be positively correlated with the concentration of primer RP2-r5 (not shown). Absence of this product in PCRs but its presence in RT–PCRs may be due to robust transcription of the corresponding gene, resulting in greater representation of the template in RNA samples relative to DNA samples, enhancing the possibility of mispriming events. The 489 bp amplicon is of no concern for multiplexed PCR assays but can serve as an additional control for RT–PCR assays. Primer sets MAMA2–INDEL-r and RP2-f1–RP2-r5 can be appropriately used together for both PCR and RT–PCRs for specific detection of the *RB* transgene.

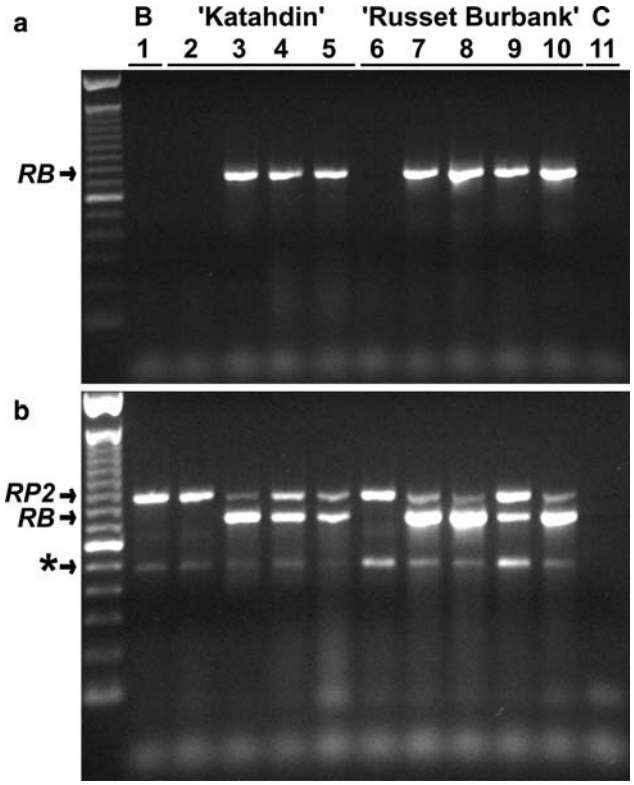

Fig. 6. Non-multiplex and multiplex RT–PCR using *RB* transgene-specific primers and *RNA Polymerase II* primers. MAMA2 and INDEL-r primers were tested in RT–PCRs (**a**) by themselves (non-multiplex reactions) and (**b**) in combination with internal control primer pair RP2 (multiplex reactions), to amplify fragments from total RNA of wild and cultivated (non-transgenic and transgenic) potato genotypes (*1 S. bulbocastanum* PT29; *2* 'Katahdin' untransformed; *3* 'Katahdin' + *RB* SP922; *4* 'Katahdin' + *RB* SP951; *5* 'Katahdin' + *RB* SP966; *6* 'Russet Burbank' untransformed; *7* 'Russet Burbank' + *RB* SP2211; *8* 'Russet Burbank' + *RB* SP2212; *9* 'Russet Burbank' + *RB* SP2213; *10* 'Russet Burbank' + *RB* SP2105; *11* H_2O control). *S. bulbocastanum* and transformed 'Katahdin' and 'Russet Burbank' have been observed to be resistant in replicated field trials (unpublished data). **a** Non-multiplex MAMA-RT–PCR. In non-multiplexed MAMA-PCRs, the *RB*-transgene product (expected fragment size: 730 bp) is detected only in the transgenic cultivated potato samples (lanes 3–5, 7–10). **b** Multiplex MAMA-RT–PCR. The *RB*-transgene product is also detected in multiplexed MAMA PCRs in only those samples that contain the *RB* transgene. The product of RP2 is amplified in all samples (expected fragment size: 862 bp), indicating this gene is present in all samples and the RT–PCRs were successful. An additional band (indicated by *asterisks*) of 489 bp was detected in all multiplexed reactions. This fragment serves as an additional control for multiplexed RT–PCRs

Discussion

In plants, R genes are commonly found in clusters of related sequences. While beneficial for isolating potential resistance gene homologs, the redundant nature of R genes complicates the molecular study of specific alleles. The *RB* late blight resistance locus is one of the five homologous sequences located on chromosome 8 of *S. bulbocastanum* (Song et al. 2003). Homologs also exist at two or more other genome locations (Pan et al. 2000a; van der Vossen et al. 2003). Development of allele-specific PCR and RT–PCR assays capable of distinguishing the *RB* transgene from other *RB* alleles and from *RB* homologs and homeologs is essential for study of *RB* transgene transcriptional regulation.

In this study, we report MAMA-PCR and RT–PCR assays specific for the *RB* transgene. Our assay results in detection of the transgene to the exclusion of the endogenous *RB* allele from which it was cloned, of other *RB* alleles (orthologs), of *RB* homologs existing within the *S. bulbocastanum RB* gene cluster and in at least two additional genome locations, and of *RB* homeologs found in cultivated potato.

A significant lesson learned in attempting to detect the *RB* transgene is the critical importance of primer design. In these assays, MAMA forward primers used in combination with a reverse primer common to known *RB* homologs do not provide sufficient specificity for the *RB* transgene. The exact reason for this is unknown, but, given the redundancy of *RB* homologs

and homeologs throughout the genome, it is possible that our MAMA forward primer might be inadvertently identical (or at least sufficiently similar) to some as yet uncharacterized *RB* homolog or homeolog. However, MAMA forward primers paired with *RB* allele- (INDEL-r) or MAMA transgene-specific (2MAMA3′1 and 2MAMA3′2) reverse primers result in amplification of only the *RB* transgene. In paired tests, MAMA primers with a mismatch in the penultimate position resulted in more specific and more robust amplification of the target sequence than MAMA primers with a mismatch in the last position, consistent with the research of Cha et al. (1992). A double MAMA approach in which both forward and reverse primers are designed to take advantage of SNPs is as specific as a MAMA forward primer paired with an *RB* allele-specific indel reverse primer. The potential to utilize a second SNP (double MAMA approach) or other target allele-specific feature for primer design may enable development of specific assays for alleles at other highly represented, clustered resistance loci.

In addition to primer design, our results indicate that PCR conditions need to be optimized. The role of dNTP concentrations in specificity has been noted previously (Creighton et al. 1992; Huang et al. 1992; Nie and Singh 2001). When using MAMA–PCR to detect *gyrA* resistance mutations in *Campylobacter jejuni*, Zirnstein et al. (1999) found that PCR conditions, including annealing and extension times and temperatures, play a critical role in the amplification of the desired allele. Even slight changes in amplification conditions can alter assay specificity. Cha et al. (1992) recommended a two-step PCR and the addition of glycerol to the reactions to enhance specificity. For the *RB* transgene, we found that modifying annealing temperature alone was sufficient to yield a highly specific assay. Furthermore, a traditional three-step PCR yielded more reliable results than did a two-step PCR. Similary, Jinneman and Hill (2001) found in their study of human pathogen *Listeria monocytogenes* that three-step PCR, with annealing temperature of 55°C, was sufficient to correctly and quickly classify isolates into lineage groups using MAMA primers. In the current study, identification of assay conditions that yield a single *RB* transgene fragment without any amplification of non-target homologs or homeologs yields the potential of adaptation of our assay to real time quantification.

Reaction conditions may be even more critical for RT–PCRs. Reverse transcriptases AMV and HIV1 have been shown to be somewhat tolerant of primer mismatches (Mendelman et al. 1990; Yu and Goodman 1992). If the primer contains a mismatch at the ultimate 3′ position of a primer, extension of the primer is more efficient by a reverse transcriptase than a DNA polymerase, when using either a DNA or RNA template (Mendelman et al. 1990; Yu and Goodman 1992). The ultimate two bases of MAMA primers are mismatched relative to the non-target template, reducing the likelihood of primer extension by either reverse transcriptase or DNA polymerase. Nevertheless, annealing temperatures and primer concentrations can be optimized to prevent non-target amplification. Although the conditions used for PCRs were easily modified for RT–PCRs in our study, additional refinements might be needed in other gene systems to adapt a MAMA–PCR assay to a MAMA–RT–PCR assay. As demonstrated in the current study, the use of a positive control in multiplexed RT–PCRs might also require optimization of primer concentrations. Since the *RB* transgene is under the control of the native *RB* promoter, the transgene is expected to be constitutively expressed at low levels. A lower *RP2* primer concentration was required for RT–PCRs compared to PCRs, suggesting a lower relative abundance of the *RB* transcript compared to the *RP2* transcript. MAMA–RT–PCR assays for other transgenes may therefore require optimization of primer concentrations depending on the strength of the transgene promoter and the relative abundance of the transgene transcript compared to that of the control gene.

MAMA primers incorporating every possible nucleotide mismatch in the penultimate position performed differently in MAMA–indel and double MAMA reactions (Fig. 5, S1). It has been reported that primers that have a purine:pyrimidine or pyrimidine:purine mismatch at the ultimate 3′ position extend more efficiently than primers terminating in either pyrimidine:pyrimidine or purine:purine mismatches (Mendelman et al. 1990; Huang et al. 1992; Burnouf and Fuchs 2000). For MAMA primers, the target-specific SNP is most frequently incorporated at the extreme 3′ nucleotide position, but the researcher must specify the nature of the penultimate mismatch. Recent MAMA applications have favored transversal (purine to pyrimidine or pyrimidine to purine) penultimate mismatches, with C being selected as the mismatch nucleotide whenever possible (e.g., Wagner et al. 1997; Buzard et al. 1999; Borucki and Call 2003). Presumably, transversions accentuate primer–template mispairing, reducing the likelihood of primer extension. However, Cebula et al. (1995) successfully utilized a transitional penultimate mismatch. Other researchers have used both transversal and transitional mismatches, in separate reactions, to amplify different

alleles (Glaab and Skopek 1999; Qiang et al. 2002; Gale and Tafoya 2004). In the current study, we tested every possible mismatch nucleotide in the penultimate position. The MAMA primers that functioned best in terms of *RB* transgene specificity and amplicon robustness were those incorporating a mismatch to C [transversion: MAMA2-A→C and 2MAMA5′3-A→C; transition: MAMA3′1-T→C (Table 1)].

To our knowledge, MAMA has not been utilized to study transgenes. Virtually, all published studies employing MAMA involve mutations important in animals. In plants, MAMA or a MAMA-like approach has been used to identify SNPs for creation of molecular markers (Drenkard et al. 2000; Hayashi et al. 2004, 2006). MAMA has also been used to manipulate BACs as an alternative to the more time-consuming use of selectable markers (Swaminathan et al. 2001). As more plant R alleles are characterized and as more transgenes are deployed for crop improvement, MAMA-based assays may serve as tools for broader study of transgenes at the DNA sequence and transcriptional levels. We demonstrate in this study that transgenes can be specifically amplified even in a complex genetic background containing multiple homologs or homeologs, facilitating the identification and study of transformed plants.

Recently, a primer set was developed to aid in marker-assisted selection of *RB* (Colton et al. 2006). For reverse primer design, the authors took advantage of the same 18 bp indel that we targeted for design of primer INDEL-r, although a distinct reverse primer was ultimately developed. Colton et al. (2006) paired their indel reverse primer with a standard forward primer developed from the *S. bulbocastanum RB* (resistance) allele. The resulting primer pair amplifies both the *RB* transgene and the endogenous *RB* allele. In the current study, we used a MAMA approach to develop PCR and RT–PCR assays that are highly specific for the *RB* transgene. Our assays have been multiplexed with a conserved internal control appropriate for both PCR and RT–PCR, providing confidence in negative (*RB* transgene amplicon absent) reactions. The development of *RB* transgene-specific assays in the current study expands upon and compliments the marker-assisted breeding resources developed by Colton et al. (2006).

Ongoing studies in our laboratory entail the adaptation of our MAMA *RB* transgene-specific assays to gel based and real time quantification. TaqMAMA, a quantitative MAMA assay in which the TaqMan® system is used in conjunction with MAMA primers, has been described and used successfully in human DNA (Glaab and Skopek 1999) and rat RNA (Bleicher et al. 2001) systems, facilitating diagnostics and allelic discrimination. The successful detection of the transgene *RB* in a quantitative manner will allow for the assessment of transgene transcription throughout plant development. When paired with phenotypic tests, these assays will allow for better correlation between transcription of the gene and late blight resistance, especially when viewed across multiple transgenic lines from different potato cultivars.

MAMA–PCR and RT–PCR are effective molecular methods for distinguishing between transgene *RB* and endogenous *RB* alleles, paralogs, homologs, and homeologs. We anticipate that the approaches detailed here will yield allele-specific PCR and RT–PCR assays for the study of other plant R genes.

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