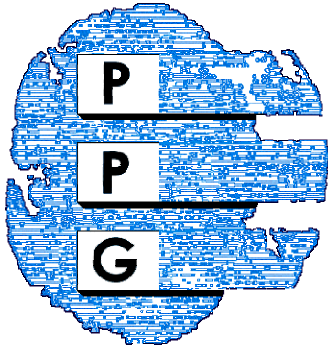

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MAMA PCR and RT-PCR assay for *RB*

Note: The following protocol was adapted from Applied Biosystems (Foster City, CA; DNA) and Invitrogen (Carlsbad, CA; RNA) literature accompanying AmpliTaq™ and SuperScript™ One-Step RT-PCR kit, respectively. It has been successfully used by the Bradeen Lab to selectively amplify a transgene from transformed lines of cultivated potato *Solanum tuberosum*. Other commercially-available *Taq* preparations and reverse transcriptase kits may work just as well as AmpliTaq™ or SuperScript™ One-Step RT-PCR kit. Mention of Applied Biosystems, Invitrogen, Promega, and their products in this protocol reflects our research experiences but should not be interpreted as endorsement to the exclusion of other products.

Justification

This assay was used to identify lines of cultivated potato transformed with the late blight resistance gene *RB*. The technique of MAMA (Mismatch Amplification Mutation Assay (Cha *et al.* 1992)) was used to selectively amplify the transgene and not homeologues in the potato genome. MAMA primers take advantage of transgene-specific SNPs introduced into late blight resistance gene *RB* by LR-PCR (Song *et al.* 2003). Additional details and results of this assay can be found in Millett and Bradeen (2007).

Primers

All primers were designed using Primer3 (http://frodo.wi.mit.edu/). MAMA primers for the gene-of-interest were generated by placing the transgene-specific SNP at the ultimate 3' position of the primer. The penultimate base of the primer was intentionally changed to one of three possible mismatch bases. The primer with the penultimate base of C was used for these assays. MAMA primers were designed to be 24-27 bases in length with a calculated melting temperature of 58-60°C. Primers generated for *RNA Polymerase II* subunit *2* (*RP2*) were based on sequence from tomato, *S. esculentum* (GenBank number 1049067), and served as an internal control*. RP2* primers generated an amplicon greater in size than the *RB* amplicon. Multiplexing with an internal control gene serves to indicate that the PCR did not fail when the gene-of-interest amplicon is not generated.

MAMA PCR

1. The following components are mixed in a thin-walled, 200µl PCR tube on ice.

5 µL 10X AmpliTaq™ buffer (supplied by Applied Biosystems)
1 µL 50x dNTP mix (=400uM final concentration)
1 µL Forward internal control gene primer (= 1 µM final concentration) *
1 µL Reverse internal control gene primer (= 1 µM final concentration) *
1 µL Forward gene-of-interest primer (= 1 µM final concentration) *†
1 µL Reverse gene-of-interest primer (= 1 µM final concentration) *†
0.5 µL AmpliTaq™ (Applied Biosystems) (= 1.25 units)
0.5 µL Template DNA (= 15 ng) ‡
<u>38 µL ddH_2O</u>
50.0 µL Total volume

* primer concentrations can be adjusted as required for sensitivity
† either or both gene-of-interest primers can be MAMA primers
‡ Isolated from frozen, ground leaf tissue using a modified cTAB protocol (Fulton *et al.* 1995)

2. Use the following thermocycler conditions with a "hot start" (i.e. begin thermocycler program, keeping reaction tube on ice until the thermocycler is >90°C; quickly and carefully place reaction tube in the thermocycler and close the lid):

1 cycle:	94°C 1'
35 cycles:	94°C 15"
	57°C 30"
	68°C 50"
1 cycle:	72°C 6'

Note: To test amplification conditions, the annealing temperature was altered by two degree increments, ranging from 51°C and 61°C.

3. Visualize the amplicons via standard gel electrophoresis (1% agarose gel in TBE buffer) and ethidium bromide staining. Samples with a positive gene-of-interest band contain the gene-of-interest.

MAMA RT-PCR

1. The following components are mixed in a thin-walled, 200µl PCR tube on ice.

25 µL 2X buffer (supplied by Applied Biosystems)
1 µL Forward internal control gene primer (= 0.25 µM final concentration) *
1 µL Reverse internal control gene primer (= 0.25 µM final concentration) *
1 µL Forward gene-of-interest primer (= 1 µM final concentration) *†
1 µL Reverse gene-of-interest primer (= 1 µM final concentration) *†
1 µL SuperScript II™ taq mix (Invitrogen)
2 µL Template RNA (= 15 ng) ‡
18 µL ddH_2O
50.0 µL Total volume

* primer concentrations can be adjusted as required for sensitivity. *RNA Polymerase II* primers were tested at 1 µM, 0.5 µM, 0.25 µM, and 0.1 µM concentrations.
† either or both gene-of-interest primers can be MAMA primers
‡ Isolated from frozen, ground leaf tissue using SV Total RNA Isolation kit (Promega, Madison, WI) according to manufacturer's recommendations

2. Use the following thermocycler conditions with a "hot start" (i.e. begin thermocycler program, keeping reaction tube on ice until the thermocycler is >45°C; quickly and carefully place reaction tube in the thermocycler and close the lid):

1 cycle:	50°C 30'
1 cycle:	94°C 2'
35 cycles:	94°C 15"
	57°C 30"
	68°C 50"
1 cycle:	72°C 6'

Note: To test amplification conditions, the annealing temperature was altered by two degree increments, ranging from 51°C and 61°C.

3. Visualize the amplicons via standard gel electrophoresis (1% agarose gel in TBE buffer) and ethidium bromide staining. Samples with a positive gene-of-interest band contain the gene-of-interest.

References

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