Modification of oligo design for enhanced sensitivity and specificity of a DNA macroarray for detection of fungal onion bulb rot pathogens

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Introduction

- Onion (*Allium cepa* L.) is an important vegetable crop in Washington State, the U.S., and many other countries.
- Losses of onion bulbs to storage rots can have a significant financial impact on the onion industry.
- Management of bulb rots is confounded by limited resources for rapid detection of bulb rot pathogens.
- A method for rapid, accurate, and sensitive detection of bulb rot pathogens in onion bulbs is needed, including latent infection of bulbs at harvest.

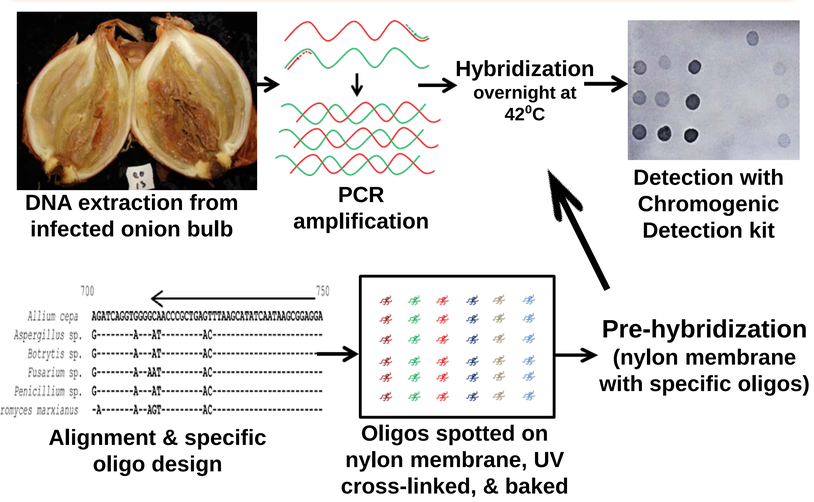
Objectives

- Development of a DNA macroarray to identify and discriminate 14 fungi and 1 yeast that can cause onion bulb rots, of which *Botrytis aclada*, *B. allii*, and *B. byssoidea* are important neck rot pathogens.
- Optimize sensitivity and specificity of the macroarray by oligo modification.

Materials and Methods

- Genus- and species-specific oligos were designed from the internal transcribed spacer (ITS) and L45-550 gene region of *Botrytis* spp. The ITS region was used to design oligos for the yeast pathogen, *Kluyveromyces marxianus.*
- Fungal and yeast isolates were grown on ½-strength V8 agar medium.
- Total genomic DNA was extracted from 3- to 7-day old cultures (UltraClean Soil DNA Isolation Kit, MOBIO, Carlsbad, CA).
- Universal primer sets for fungi (ITS5/ITS4A) and *Botrytis* (BA2f/BA1) were multiplexed in a single reaction for PCR amplification. Biotin-14-dATP and regular dNTPs were used in the PCR reaction to label amplicons.
- PCR conditions: initial denaturation at 95^{0}C for 3 min; followed by 40 cycles with denaturation at 95^{0}C for 30 s, annealing at 52^{0}C for 1 min, extension at 72^{0}C for 1 min; with a final extension at 72^{0}C for 3 min.
- Amersham Rapid-hyb buffer (GE Healthcare, Buckinghamshire, UK) and SSC hyb-buffer (6X SSC, 5X Denhardt's solution, 0.5% SDS and 50% deionized formamide) were evaluated.

Procedure



Genus- and Species-Specific Oligos

Table 1. Oligos designed for a macroarray to detect and discriminate *Botrytis* spp. and *Kluyveromyces marxianus.*

Target genus/species	Name	Sequence	Region
Botrytis	BotGen	GCTTGGTATTGAGTCTATGTCAGTAATG	ITS
	Bt3f	AGTAGATGGTCGAAGGAACGCTCTC	L45-550
	Bot (+)	TGCGCCCGCCAGTATTCTG	ITS
Botrytis aclada	B.acl UP-1	CGCATATATTTTAGTAAAATGGACCTCAC	L45-550
	Btacl1	CGTTTTTCGGTGACTCATATGTC	L45-550
Botrytis byssoidea	B.bys UP-3	CTCGTGCTGTTTGCGCAGATAAT	L45-550
Botrytis cinerea	B. cin UP-3	CTACCACAAATAGCTACAACATACCTTCA	L45-550
Kluyveromyces marxianus	Klu K2 v1	ACCTTTGGGTTTGGTAGTGAGTGATAC	ITS
	KlumF	TTTTCGTGAATCATCAAATCTTTG	ITS
	KluMGen	GTATTGTGAATTGCAGATTTTCGTGAA	ITS
All fungi/yeast	*ITS 4A	ATGCTTAAGTTCAGCGGGTA	ITS
	**BA2f	GTGGGGGTAGGATGAGATGATG	L45-550

ITS rDNA sequences for >120 fungal isolates associated with onions were aligned with CLUSTALW to identify polymorphisms. Oligonucleotide probes were designed with Lasergene Core Suite to unique DNA regions common to pathogenic isolates. Universal primer ITS4A and primer BA2f = positive controls for all fungi and the yeast. *Modified ITS4 primer (White et al. 1990). **Nielsen et al. (2002).

Oligo Modifications

Each oligo was modified by addition of a customized nucleotide sequence at 5' and/or 3' terminus:

a) Oligo with no modification was cross-linked on nylon membrane

5'-GTGGGGGTAGGATGAGATGATG-3'

BA2f: Tm 57^{0}C; Length 22; GC 55%

b) Oligo with a customized tail at 5' terminus to raise the Tm up to 62^{0}C was cross-linked on nylon membrane

5'-TTTTTCTTTTTTCTTTT-GTGGGGGTAGGATGAGATGATG-3'

BA2f-M2: Tm 62^{0}C; Length 39; GC 36%

c) Oligo with customized tail at 5' and 3' termini to raise the Tm up to 65^{0}C was cross-linked on nylon membrane

5'-TTTTTCTTTTTTCTTTT-GTGGGGGTAGGATGAGATGATG-TTTTTCTTTTTTCTTTTTTT-3'

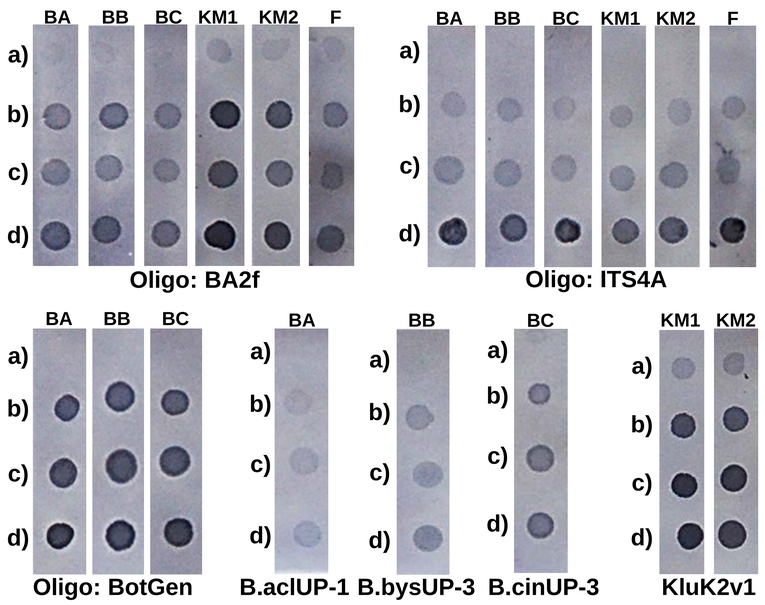
BA2f-M3: Tm 65^{0}C; Length 60; GC 27%

d) Oligo with customized long tail at 5' terminus to raise the Tm up to 65^{0}C was cross-linked on nylon membrane

5'-TTTTTCTTTTTTCTTTTTTTTTTTTTTCTTTTTTCTTTT-GTGGGGGTAGGATGAGATGATG-3'

BA2f-M4: Tm 65^{0}C; Length 60; GC 27%

Macroarray results using the modified oligos



a) no oligo modification; b), c), & d): oligos modified at 5' and/or 3' terminus as noted above; BA = *Botrytis aclada*; BB = *Botrytis byssoidea*; BC = *Botrytis cinerea*; KM1 = *Kluyveromyces marxianus* isolate Klu96; KM2 = *K. marxianus* isolate BKS2010#1; F = *Fusarium proliferatum* isolate 142-2.

Oligo Specificity

- Each oligo was analyzed *in silico* for specificity.
- Each oligo was tested against isolates of other *Botrytis* species and the genera *Fusarium, Aspergillus* and *Penicillium* for cross reactivity.

Table 2. Oligo specificity with and without modification.

Target	Oligo	B651	BB1	BA16	Klu 96	BKS2010 #1	Asp N5	Fus 142-2	Pen 66
Oligos with 5' terminus modification									
Botrytis	BotGen	+	+	+	-	-	-	-	-
	Bt3f	+	+	+	-	-	-	-	-
	Bot (+)	--	+	--	-	-	-	-	-
B. aclada	B.acl UP-1	-	-	+	-	-	-	-	-
	Btacl1	-	-	+	-	-	-	-	-
B. byssoidea	B.bys UP-3	-	+	-	-	-	-	-	-
B. cinerea	B. cin UP-3	+	+	+	-	-	-	-	-
K. marxianus	Klu K2 v1	-	-	-	+	+	-	-	-
	KlumF	-	-	-	+	+	-	-	-
	KluMGen	-	-	-	+	+	-	-	-
All fungi/yeast	*ITS 4A	+	+	+	+	+	+	+	+
	**BA2f	+	+	+	+	+	+	+	+
Oligos with no modification									
Botrytis	BotGen	--	--	--	-	-	-	-	-
	Bt3f	--	--	--	-	-	-	-	-
	Bot (+)	+	+	+	-	-	-	-	-
B. aclada	B.acl UP-1	-	-	--	-	-	-	-	-
	Btacl1	-	-	--	-	-	-	-	-
B. byssoidea	B.bys UP-3	-	--	-	-	-	-	-	-
B. cinerea	B. cin UP-3	+	-	-	-	-	-	-	-
K. marxianus	Klu K2 v1	-	-	-	+	+	-	-	-
	KlumF	-	-	-	+	+	-	-	-
	KluMGen	-	-	-	+	+	-	-	-
All fungi/yeast	*ITS 4A	--	--	--	--	--	--	--	--
	**BA2f	+	+	+	+	+	+	+	+

BA16 = *Botrytis aclada*; BB1 = *B. byssoidea*; B651 = *B. cinerea*; Klu96 = *Kluyveromyces marxianus*; BKS2010#1 = *K. marxianus*; AspN5 = *Aspergillus niger*; Fus142-2 = *Fusarium proliferatum*; Pen66 = *Penicillium glabrum*. Red indicates a false negative or false positive result. Results with oligo modifications b and c are not presented.

- Amersham Rapid-hyb buffer resulted in dark spots, but sometimes also resulted in non-specific hybridization.
- Pre-hybridization with SSC hyb-buffer followed by hybridization with mix hyb-buffer (50% Amershan Rapid-hyb buffer + 50% SSC hyb-buffer) produced the most specific results with dark spots on the membrane.

Conclusions and Future Directions

- Genus- and species-specific DNA oligos for *Botrytis* and *B. aclada, B. byssoidea, B. cinerea*, and *K. marxianus* were evaluated with PCR amplicons generated from target isolates.
- Oligos with a customized long tail at the 5' terminus (Tm 65^{0}C) worked best for almost all nucleotide sequences evaluated, resulting in enhanced specificity, and increased spot intensity on the macroarray.
- Oligos are being designed for *Fusarium*, *Aspergillus*, and *Penicillium* spp. as well as *B. allii*, which cause bulb rots.
- In an allied project, we are developing a macroarray for detection of 12 bacteria capable of causing bulb rot.

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