Development of DNA Arrays for Diagnosis and Prediction of Almond Diseases

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Interpretive Summary:

Here we report on development of a macro array (an array of diagnostic DNA probes on 115 x 75 mm nylon membranes) for almond soil pathogens. We chose to use genomic DNA (total DNA, including genes and the regions between them) from the organisms to be detected as the probes fixed on the membranes. This approach of using a macro array with genomic DNA for probing recently was reported to sensitively and specifically detect different species of sheep rumen bacteria. In theory at least, the approach affords high sensitivity and specificity without the large development costs and time required for development of comprehensive oligonucleotide arrays. To test a genomic macro array for almond we extracted DNA from two isolates of *Cylindrocarpon* sp. and single isolates of *Trichoderma* sp., *Colletotrichum acutatum*, *Verticillium dahliae*, *Phytophthora cactorum*, *P. citricola, P. cinnamomi,* and *P. megasperma*. Once fixed on

the macro array, the DNA of these pathogens was used to “probe” experimental samples containing their DNA in known amounts. These experimental samples were meant to represent extracts from a test soil or diseased plant part. It was found that the array could detect all of the pathogen DNA at reasonably low amounts. It distinguished Phytophthora pathogens from the other pathogens, but it could not reliably distinguish between different *Phytophthora* spp., and it could not reliably distinguish between all of the other fungi. Similar challenges were met in the development of the array for sheep rumen bacteria by optimizing probe concentrations on the array and annealing temperatures used to hybridize with the test samples. We are testing such optimizations for the almond pathogen array.

Background and Objectives:

Of the many methods available for detection of plant pathogens, only array-based methods afford specific detection and quantification of many microbes simultaneously. Array technology uses DNA probes (i.e., either short DNA fragments--typically 20 to 100s of base pairs long - or genomic [total] DNA from the target organisms, one desires to detect). The probes are arrayed in a known pattern on a solid surface (i.e., a nylon membrane, glass slide, or plastic chip). Each position in the array is allocated to a spot of an individual DNA probe that is specific for (i.e., will bind only to) complementary DNA from a single class or species of organism (the specificity of DNA probes varies depending on their nature). Such arrays may be used as follows: DNA extracted from a sample of interest (i.e., from a root, soil, or microbial culture) is either hybridized directly or first subjected to PCR to amplify target DNA (i.e., DNA for which the array’s DNA probes are designed to detect), then hybridized. In either case, the target (sample) DNA is labeled with a dye that will fluoresce so that it can be detected after it has hybridized to the probes. The labeled DNA is allowed to hybridize with the probes on the array. The array is then rinsed, so that the labeled target DNA will be retained (and fluoresce) only over the spots of array probes that complement it (i.e., bind it due to complementary DNA sequence). The binding specificity can be used to distinguish between individual genera, or, in some cases, species and thereby replace the need for costly sequencing.

Array formats vary tremendously, each with pros and cons (Greene and Voordouw, 2003; Levesque et al., 1998; Lievens and Thomma, 2005; Sessitsch et al., 2006). Two key areas in which array formats differ include: 1) the basic platform of the array [i.e., platforms include a) a macroarray format, in which DNA probes are arrayed using inexpensive hand-held replicators on a porous membrane (Krause et al., 2004; Le Flock et al., 2007; Lievens et al., 2003, 2005; Zhang et al., 2007, 2008); and b) a microarray format in which the probes are arrayed using expensive robotic replicators on a glass slide or with a proprietary photolithography process on a chip (Sessitsch et al., 2006; Wu et al., 2004, 2006)] and 2) the nature of the DNA probes (spots) that are fixed on the array [i.e., arrays tend to use either: a) relatively short diagnostic nucleotide fragments 20 to 100 base pairs long that, singly or in conjunction with each other, can detect specific organisms of interest (Izzo and Mazzola, 2009; Zhang et al., 2007, 2008) or b) whole-genome (total) DNA of organisms of interest (Krause et al., 2004; Wu et al., 2004, 2006)]. As described below, we have chosen to develop a nylon-membrane-based macroarray system that uses spots of whole-genome

DNA as the probe system (i.e., in our case the genomic probes were from plant pathogenic species associated with occurrence or suppression of PRD and other almond diseases). The macroarray-genomic DNA format was chosen because if offers the best potential for species-level resolution, detection sensitivity, and unbiased quantification of targets at a reasonable development cost. The chosen format is affordable, flexible, and readily expandable. The feasibility and utility of the approach we have chosen has been demonstrated (Krause et. al., 2004).

The development and application of array would benefit the almond and allied industries in two key ways. First, it would provide an adaptable and affordable tool with which to repeatedly monitor microbial community shifts associated with PRD incidence and treatments to control it. Second, it would be affordable and useful for detecting and quantifying specific, aggressive pathogens including species of *Phytophthora*, *Verticillium*, and plant parasitic nematodes in soil and tissue samples. We consider it likely that the arrays could be used predicatively (i.e., to assess the need for soil remediation treatment) as well as diagnostically (i.e., to determine the cause[s] of soilborne problems). Objectives of the project were: (1) to develop diagnostic DNA arrays that detect key soilborne almond pests and pathogens and (2) to develop diagnostic DNA arrays that characterize and identify members of soil-borne microbial communities mediating almond replant disease.

Materials and Methods:

Fungal isolates and DNA extraction

DNA was extracted from nine fungal and oomycete species and two peach rootstocks for spotting on nylon membranes and labeling with digoxigenin-11-dUTP. Fungi that were chosen for the initial phase of macro array analysis included two isolates of *Cylindrocarpon* sp. and single isolates of *Trichoderma* sp., *Colletotrichum acutatum*, and *Verticillium dahliae*. Chosen oomycetes were *Phytophthora cactorum*, *P. citricola, P. cinnamomi,* and *P. megasperma*. Mycelia were obtained by growing the fungi and oomycetes for 4 to 7 days in potato dextrose broth and V8 juice broth, respectively. Leaves of tissue culture grown plants of peach rootstocks Nemaguard and Lovell, frozen at -80^0 C for over 2 years, were used for DNA extraction. Total nucleic acids (both DNA and RNA) from all samples were extracted using a modified CTAB method (Bhat and Browne, 2009). Samples were incubated with RNaseA at 65^0 C for 10 min to degrade RNA. Purified DNA was assessed for quality and quantity by measuring the absorbance at 230, 260, and 280 nm in a NanoDrop. All DNA samples were stored at -20^0 C.

Spotting DNA on Nylon Membrane

In the first set of whole genome macro arrays, DNA suspensions (approximately 800 ng/µl) were aliquoted in each of four randomly chosen wells of a 96-well plate and heated to 95^0 C for 3 min followed by chilling on ice for 3 min. Denatured DNA was diluted 50:50 with 2X spotting buffer and mixed. Positively charged Hybond-N^+ nylon membranes (75 mm x 115 mm) were soaked in 10X SSC before 0.5 µl of each target sample was spotted using a 96-slot pin replicator. Spotted membranes were placed with DNA-side up on blotting papers soaked in denaturing solution for 5 min and then on

blotting papers soaked in neutralizing solution for 1 min, followed by air-drying for 1 hr at room temperature. The DNA was fixed to the spotted membranes by exposing to UV lamps at 120mJ/cm^2 for 65 sec in a UV cross linker. Dried and fixed membranes were stored in a zip lock bag at 4^0 C. By using the same DNA spotting procedure, 12 concentrations (50 ng to 10 pg) of eight target DNAs were spotted on each membrane in a second set of whole genome macro arrays.

Nick Translation

To label the sample DNA (i.e., the DNA of the target pathogens in experimental samples) 2 µl of DIG-Nick Translation Mix (Roche Diagnostics GmbH, Mannheim, Germany) were mixed with approximately 500 ng of sample DNA in 8 µl suspension. After incubating the mixture at 15^0 C for 90 min in an incubator, the reaction was stopped by adding 0.5 µl of 0.5M EDTA (pH 8.0) and incubating at 65^{0}C for 10 min. Digoxigenin (DIG)-labeled DNA samples were stored at -20^0 C

Hybridization and Stringency Washing:

The spotted membrane was placed in a large rolling tube (80 mm in diameter) with DNA spots facing inside of the tube (i.e., side of the membrane with DNA spots not adhering to the glass surface) and 10 ml of Pre-hybridization Buffer was added. The membrane was incubated with gentle agitation at the desired temperature (60, 65 or 70^0 C) for 1 hr in a Hybridizer. DIG-labeled DNA sample was denatured by heating at 95^{0}C in a heating block for 10 min and immediately chilling on ice for 5 min. To obtain hybridization buffer, 1 µl of denatured labeled DNA (50 ng) was added to 10 ml of pre-hybridization buffer that was pre-warmed at the desired temperature (60, 65 or 70^0 C) in a water bath. The membrane was incubated overnight in 10-ml Hybridization Buffer at the desired temperature (60, 65 or 70^0 C). After hybridization, the membrane in the roller tube was washed twice by gentle agitation in a Hybridizer with 50 ml of Low Stringency Buffer at 25^0 C for 15 min, followed by two washings with 50 ml of pre-warmed High Stringency Buffer at the desired temperature (60, 65 or 70^0 C) for 15 min.

Blocking, Antibody Binding and Detection**:**

After the high stringency washing, the membrane was rinsed in 50-ml Washing Buffer at room temperature for 5 min with gentle shaking on an orbital shaker. Then the membrane was incubated in 50 ml of freshly prepared 1X Blocking Buffer at room temperature for 30 min with gentle shaking. After discarding the Blocking Buffer, the membrane was transferred to a zip lock bag containing 2 µl of stock Anti-Digoxigenin-AP Fab fragments (750 mU/µl) (Roche Diagnostics GmbH, Mannheim, Germany) in 20 ml of pre-warmed 1X Blocking Buffer (i. e., 1:10,000 dilution). The zip lock bag was sealed after removing air bubbles and incubated at room temperature for 30 min with gentle shaking.

The antibody-bound membrane was washed twice in 50 ml of Washing Buffer for 15 min with gentle shaking, followed by equilibration in 50 ml of Detection Buffer for 5 min. The membrane was placed on a Saran wrap (12" x 12") with the DNA side up and 1 ml of CDP-Star® substrate solution (Applied Biosystems, Foster City, CA) was distributed uniformly on the membrane. The membrane was covered with the Saran wrap by carefully folding over it, and the edges were sealed. Afterwards, the membrane was

incubated at room temperature for 5 min with gentle shaking and then at 37^0 C for 10 min without shaking in an incubator. The excess liquid with CDP-*Star*® solution was taken off from the membrane by making a small opening at the corner of Saran wrap seal, and the sealed membrane was exposed to Kodak X-ray films inside a cassette for 5 and 30 min before developing the films.

Results and Discussion:

The purity of genomic DNA used, after appropriate dilutions, for probing and preparation of experimental samples was good. Initial fungal DNA concentrations varied between 31.8 to 284.3 ng/µl with an average ratio of 1.95 for A_{260}/A_{280} and 1.35 for A_{260}/A_{230}. For *Phytophthora* spp., DNA concentrations varied between 106.0 to 317.4 ng/µl with an average ratio of 2.03 for A_{260}/A_{280} and 2.17 for A_{260}/A_{230}. For Nemaguard and Lovell, the DNA concentration was 1047.0 ng/µl with a ratio of 1.84 for A_{260}/A_{280} and 1.29 for A_{260}/A_{230} and 1199.1 ng/µl with a ratio of 1.82 for A_{260}/A_{280} and 1.19 for A_{260}/A_{230}, respectively. For all 11 target DNA samples, the concentration was adjusted to approximately 800 ng/µl, and they were spotted on nylon membranes with 200 ng DNA per spot. Spots with sterile distilled water served as a negative control.

In general, DIG-labeled whole genomic DNA hybridized well with target whole genomic DNA on nylon membranes. In the first set of macroarray membranes, cross hybridization occurred among genomic DNA of four *Phytophthora* spp. and between genomic DNA of two peach rootstocks. Labeled whole genomic DNA of *Phytophthora* spp. or peach rootstocks did not hybridize with *Cylindrocarpon* sp., *C. acutatum*, *Trichoderma* sp., or *V. dahliae* and vice versa. However, labeled whole genomic DNA of *Cylindrocarpon* sp., *C. acutatum*, *Trichoderma* sp., or *V. dahliae* hybridized non-specifically among their probes. The pattern of hybridization was similar at temperature levels of 60^0, 65^0 and 70^0 C. Usually the 30-min exposure of X-ray films resulted in higher spot intensities as compared to 5-min exposure. At any of the hybridization temperatures, none of the labeled whole genomic DNA of target organisms hybridized with water only (negative control) spots, indicating the absence of background hybridization.

In the second macro array experiment, in which hybridization temperatures tested were 60^0, 65^0 and 70^0 C, probe DNA amounts fixed on the array were 50 ng, 25 ng, 10 ng, 5 ng, 2 ng, 1 ng, 500 pg, 200 pg, 100 pg, 50 pg, 25 pg, or 10 pg per spot. Specificity and sensitivity generally were not improved over the levels obtained in the first experiments (**Table 1**). That is, the array distinguished between DNAs from *Phytophthora*, peach, and the fungi in the experimental samples, but it could not distinguish among DNAs from different species of Phytophthora, different genera of the fungi or the two selections of peach (**Table 1**). The intensity of hybridization was proportional to the amount of DNA per spot and the exposure time. The optimum amount of DNA per spot for 5-min exposure to X-ray films was 200 ng, and obtaining this high amount of DNA (i. e. 800 ng/µl) for all target organisms is cumbersome, hence a disadvantage to attain assay sensitivity.

Our results to date are requiring us to examine alternative approaches to development of the diagnostic capabilities needed at the same time as we exhaust test further optimizations of the genomic macro array.

Recent Publications, Literature Cited:

Bhat, R. G., and Browne, G. T. 2009. Specific detection of *Phytophthora cactorum* in diseased strawberry plants from nurseries and fruiting fields by nested polymerase chain reaction. (Accepted for publication in *Plant Pathology*)

Browne, G.T., Connell, J.H., and Schneider, S.M. 2006. Almond replant disease and its management with alternative pre-plant soil fumigation treatments and rootstocks. Plant Disease 90:869-876.

Greene, E. A., and Voordouw, G. 2003. Analysis of environmental microbial communities by reverse sample genome probing. Journal of Microbiological Methods 53:211-219.

Izzo, A. D., and Mazzola, M. 2009. Hybridization of an ITS-based macroarray with ITS community probes for characterization of complex communities of fungi and fungal-like protists. Mycological Research 113:802-812.

Krause, D.O., Smith, W.J.M., McSweeney, C.S. 2004. Use of community genome arrays (CGAs) to assess the effects of *Acacia angustissima* on rumen ecology. Microbiology 150:2899-2909.

Le Floch, G., Tambong, J., Vallance, J., Tirilly, Y., Levesque, A., and Rey, P. 2007. Rhizosphere persistence of three *Pythium oligandrum* strains in tomato soilless culture assessed by DNA macroarray and real-time PCR. FEMS Microbiology and Ecology 61:317-326.

Levesque, C.A., Harlton, C. E., and de Cock, A. W. A. M. 1998. Identification of some oomycetes by reverse dot blot hybridization. Phytopathology 88:213-222.

Lievens, B., and Thomma, B. P. H. J. 2005. Recent developments in pathogen detection arrays: Implication for fungal plant pathogens and use in practice. Phytopathology 95:1374-1380.

Lievens, B., Brouwer, M., Vanachter, A. C. R. C., Levesque, A., Cammue, B. P. A., and Thomma, B. P. H. J. 2003. Design and development of a DNA array for rapid detection and identification of multiple tomato vascular wilt pathogens. FEMS Microbiology Letters 223:113-122.

Lievens, B., Brouwer, M., Vanachter, A. C. R. C., Levesque, A., Cammue, B. P. A., and Thomma, B. P. H. J. 2005. Quantitative assessment of phytopathogenic fungi in various substrates using a DNA macroarray. Environmental Microbiology 7:1698-1710.

Sessitsch, A., Hackl, E., Wenzl, P., Kilian, A., Kostic, T., Stralis-Pavese, N., Sandjong, B. T., and Bodrossy, L. 2006. Diagnostic microbial microarrays in soil ecology. New Phytologist 171:719-736.

Wu, L, Liu, X., Schadt, C. W., and Zhou, J. 2006. Microarray-based Analysis of subnanogram quantities of microbial community DNAs by using whole-community Genome Amplification. Applied and Environmental Microbiology 72:4931-4941.

Wu, L., Thompson, D.K., Liu, X., Fields, M.W., Bagwell, C.E., Tiedje, J.M., Zhou, J. 2004. Development and evaluation of microarray-based whole-genome hybridization for detection of microorganisms within the context of environmental applications. Environmental Science and Technology 38:6775-6782.

Zhang, N., Geiser, D. M., and Smart, C. D. 2007. Macroarray detection of solanaceous plant pathogens in the *Fusarium solani* species complex. Plant Disease 91:1612-1620.

Zhang, N., McCarthy, M. L., and Smart, C. D. 2008. A macroarray system for the detection of fungal and oomycete pathogens of solanaceous crops. Plant Disease 92:953-960.

Table 1. Specificity and sensitivity results, development of nylon membrane-based macroarray for almond pathogens

Genomic DNA arrayed on Hybond^{+} Nylon membrane	Annealing temp (^{0} C)	Minimum amount of genomic probe DNA (ng) required to hybridize with target DNA[b]											
		P. cactorum		*P. citricola*		*P. cinnamomi*		*P. megasperma*		Nemaguard		Lovell	
		5 min[a]	30 min	5 min	30 min	5 min	30 min	5 min	30 min	5 min	30 min	5 min	30 min
P. cactorum	60	25	25	-	25	-	50	-	-	-	-	-	-
	65	25	10	-	-	-	-	-	-	-	-	-	-
	70	25	25	-	25	-	-	-	-	25	25	25	25
P. citricola	60	25	25	25	10	50	25	-	-	-	-	-	-
	65	50	25	25	5	-	25	-	50	-	-	-	-
	70	5	2	5	2	25	5	-	25	50	50	50	50
P. cinnamomi	60	-	25	-	10	50	5	-	-	-	-	-	-
	65	25	10	-	25	25	5	-	-	-	-	-	-
	70	25	10	10	5	5	2	-	25	-	-	-	-
P. megasperma	60	-	-	-	-	-	-	-	-	-	-	-	-
	65	50	25	-	50	-	-	-	-	-	-	-	-
	70	-	-	-	-	-	-	-	-	-	-	-	-
C. acutatum	60	-	-	-	-	-	-	-	-	-	-	-	-
	65	-	-	-	-	-	-	-	-	-	-	-	-
	70	-	-	-	-	-	-	-	-	-	-	-	-
Trichoderma sp.	60	-	-	-	-	-	-	-	-	-	-	-	-
	65	-	-	-	-	-	-	-	-	-	-	-	-
	70	-	-	-	-	-	-	-	-	-	-	-	-
Nemaguard	60	-	-	-	-	-	-	-	-	-	25	-	-
	65	-	-	-	-	-	-	-	-	-	25	-	25
	70	-	-	-	-	-	-	-	-	25	2	25	5
Lovell	60	-	-	-	-	-	-	-	-	25	25	-	25
	65	-	-	-	-	-	-	-	-	25	0.01	25	10
	70	-	-	-	-	-	-	-	-	-	25	-	25

[a] Exposure time of X-ray films to antibody-bound membranes that were covered with CDP-*Star* substrate solution.
[b] - = No hybridization detected.