Advances in Diagnostics of Downy Mildews: Lessons Learned from Other Oomycetes and Future Challenges

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Downy mildews are plant pathogens that damage crop quality and yield worldwide. Among the most severe and notorious crop epidemics of downy mildew occurred on grapes in the mid-1880s, which almost destroyed the wine industry in France (Gessler et al. 2011). Since then, there have been multiple outbreaks on sorghum and millet in Africa (Jeger et al. 1998), tobacco in Europe (Zipper et al. 2009), and recent widespread epidemics on lettuce, basil, cucurbits, and spinach throughout North America (Cohen and Ben-Naim 2016; Correll et al. 2011; Parra et al. 2016). In the mid-1970s, loss of corn to downy mildew in the Philippines was estimated at US\$23 million (USDA 2013). Today, crops that are susceptible to downy mildews are worth at least \$7.5 billion of the United States' economy (USDA 2015). Although downy mildews cause devastating economic losses in the United States and globally, this pathogen group remains understudied because they are difficult to culture and accurately identify.

Early detection of downy mildews in the environment is critical to establish pathogen presence and identity, determine fungicide resistance, and understand how pathogen populations disperse. Knowing when and where pathogens emerge is also important for identifying critical control points to restrict movement and to contain populations (Fisher et al. 2012). Reducing the spread of pathogens also decreases the likelihood of sexual recombination events and discourages the emergence of novel virulent strains. A major challenge in detecting downy mildews is that they are obligate pathogens and thus cannot be cultured in artificial media to identify and maintain specimens (Hall 1996). However, advances in molecular detection techniques hold promise for rapid and in some cases, relatively inexpensive diagnosis. In this article, we discuss recent advances in diagnostic tools that can be used to detect downy mildews. First, we briefly describe downy mildew taxonomy and genetic loci used for detection. Next, we review issues encountered when identifying loci

and compare various traditional and novel platforms for diagnostics. We discuss diagnosis of downy mildew traits and issues to consider when detecting this group of organisms in different environments. We conclude with challenges and future directions for successful downy mildew detection.

The Downy Mildews

Downy mildews are part of the kingdom Chromista, subphylum Oomycota (commonly oomycetes), and are within the family Peronosporaceae (Thines 2014). Downy mildews are classically identified by observations of sexual (e.g., antheridia and oogonia) and asexual structures (e.g., sporangia) using dichotomous keys. However, not all of the necessary structures for identification are observed when using light microscopy, rendering visual identification problematic. The host matrix can also induce radically different pathogen morphologies, especially for downy mildew species that have broad host ranges (Runge and Thines 2011; Runge et al. 2012). Moreover, a number of recently described species are not included on available keys such as *Peronospora somniferi*, the cause of downy mildew in opium poppy (Voglmayr et al. 2014), which was previously categorized as *P. arborescens* (Thines and Choi 2016). Due to their obligate nature, downy mildews cannot be grown on artificial media, and require frequent transfers onto susceptible host tissue. Classification of downy mildews is somewhat artificial because it relies on the perceived host range for identification, which can be especially problematic for closely related species such as *Pseudoperonospora cubensis* and *P. humili* (Choi et al. 2005b; Runge and Thines 2012). Additional challenges exist when identifying downy mildews because their phylogenetic relationships are refined continuously as we learn more about their evolution through DNA sequencing (Göker et al. 2007; Judelson 2012; Thines and Choi 2016).

Several common genera of downy mildew cause widespread disease in crops and ornamentals. Typically, host specificity is observed at the species level, whereas at the genus level, the host range is much broader. For example, *Bremia* has been known to infect different tribes in the Asteraceae but *Bremia lactucae* is restricted to lettuce (*Lactuca sativa*) and prickly lettuce (*L. serriola*) (Thines et al. 2010) (Fig. 1A). *Hyaloperonospora* spp. cause disease in broccoli, Brussels sprouts, cabbage, and other cruciferous vegetables (Coelho et al. 2012). *Peronospora* spp. attack basil, soybeans, onion, spinach,

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tobacco, and flowers such as impatiens and snapdragons (Thines and Choi 2016) (Fig. 1B and C). *Plasmopara* spp. causes downy mildew in grapes and sunflower (Gessler et al. 2011; Mestre et al. 2016) (Fig. 1D). *Pseudoperonospora* spp. can infect hops and cucurbits (Runge and Thines 2012) (Fig. 1E and F). *Sclerophthora* spp. causes downy mildew of wheat, corn, rice, and sorghum (Abad et al. 2013) and *Sclerospora* spp. causes disease in millets and other grasses (Babu and Sharma 2015). Resolving taxonomies and identifying host specificity is further complicated by cryptic species that are not well recognized as in the case of cucurbit and sunflower downy mildew (Rivera et al. 2016). Advances in molecular diagnostics together with phylogenetics can help clarify downy mildew taxonomy, host range, and specificity.

Identifying Loci and Developing Markers for Downy Mildew Detection

Due to the challenges associated with morphological identification and the fact that some downy mildew taxa can have multiple hosts, the availability of a molecular technique to rapidly and accurately identify taxa would provide a non-subjective means for isolate classification. Of particular importance is the development of amplification primers that would be specific for amplification of downy mildew sequences without the interference of nontarget amplification. Several loci have been commonly used for phylogenetic purposes and may have utility for sequenced based identification; for example, the large ribosomal subunit of the ribosomal DNA (rDNA), internal transcribed spacer region (ITS) of the rDNA, *hsp90*, *cox2*, *cox1*, *nad1*, *rps10* (Choi et al. 2011, 2015a, b; Thines et al. 2009; Voglmayr et al. 2004). Choi et al. (2015a) observed that *cox2* was more useful as a bar code for identification of downy mildews than *cox1*. Another locus that can be useful for this is the mitochondrially encoded *rps10* gene. Amplification primers for this locus were developed in highly conserved mitochondrial tRNA coding regions and were used to clarify the phylogeny of *Phytophthora* (Martin et al. 2014), but

subsequent examination found that in oomycetes there is an unusual gene order of *tRNA-F* and *tRNA-K* genes flanking either side of the *rps10* gene that has not been observed in plants or Eumycotan fungi, thereby improving specificity for oomycete amplification and reducing potential problems with nontarget amplification of this locus (F.N. Martin, unpublished). Choi et al. (2015b) observed amplification with this locus from a range of *Peronospora* spp. and amplification from *Bremia*, *Peronospora*, *Peronosclerospora*, and *Pseudoperonospora* species has also been observed (F. N. Martin, unpublished); based on sequence analysis of the mitochondrial genome, *Hyaloperonospora*, *Plasmopara*, and *Sclerospora* species should be amplified as well. It should be noted that some closely related taxa may not be differentiated; while *Pseudoperonospora cubensis* and *P. humuli* can be, *Peronospora effusa* and *P. schachtii* have identical sequences (F. N. Martin, unpublished).

It is important that diagnostic markers be highly specific and sensitive enough to detect the pathogen when it is present in low amounts. From a diagnostic standpoint, it is also helpful if detection is done at a genus level (in addition to a species-specific level), thereby enabling the identification of a broader range of species that may be present (Bilodeau et al. 2014; Miles et al. 2015, 2017). Another factor that can improve the reliability of the assay across different labs is to not rely strictly on high stringency during amplification to ensure specificity. Designing assays targeting unique sequences (Kunjeti et al. 2016) or a unique gene order (Bilodeau et al. 2014; Miles et al. 2015, 2017) will reduce the error rate when samples are run on thermal cyclers with different ramping times or are calibrated differently.

To improve detection sensitivity, marker systems are often designed based on high copy number targets such as the rDNA or the mitochondrial DNA, but if pathogen quantification is also desired, there are several considerations to keep in mind. For example, rDNA varies in copy number in Eumycotan fungi (reviewed in Martin et al. [2012]), among isolates of *Phytophthora vexans* (Spies et al. 2011), and this may also occur in *Pythium* (Schroeder et al. 2013); it is unknown if this is a characteristic of oomycetes generally. Likewise, it is unknown at this time if mitochondrial copy number varies among isolates, species, or physiological stage of infection (early stage lesion compared with older senescent lesion). For these reasons, reliability of both loci to accurately quantify the pathogen should be experimentally verified prior to using data for quantification purposes.

One of the most important aspects of marker development is proper validation. In order to validate an assay, it is important to fully evaluate specificity with a geographically diverse group of isolates of the target species to ensure all will be detected by the assay as well as a range of closely related species to evaluate if there will be any non-specific background detection. Having a robust sequence dataset of the locus representing the breadth of the genus and knowledge of the phylogenetic relationships within the genus will facilitate this effort (Bilodeau et al. 2014; Martin et al. 2014; Miles et al. 2015). It is important to test these related species to confirm specificity and to then perform validations with field samples to ensure there are no problems with cross reactivity in plant or environmental samples (e.g., confirming positive results with sequence analysis). Once an assay is developed, there should be validation in multiple laboratories to ensure the assay can consistently provide accurate detection. It is also helpful to have an evaluation where the samples are provided blind for validation of specificity. For example, there was a ring trial to evaluate specificity of available diagnostic assays for *P. ramorum* using a standardized library of DNA from a wide range of species (Martin et al. 2009).

Advantages and Disadvantages of Different Loci Used for Downy Mildew Pathogen Detection

Choosing the best locus for molecular diagnostics is one of the most challenging aspects in marker development and there are multiple issues to consider. For molecular detection of downy mildew pathogens, the primary locus has been the ITS region, and assays have been reported with varying levels of validation for several

Fig. 1. Sporulation of six economically important downy mildew pathogens discussed in this review. **A**, *Bremia lactucae* on lettuce; **B**, *Peronospora belbahrii* on basil; **C**, *Peronospora effusa* on spinach; **D**, *Plasmopara viticola* on 'Niagara' grapes; **E**, *Pseudoperonospora cubensis* on cucumber; and **F**, *Pseudoperonospora humuli* on hops. Photo credits to Steve Koike (University of California) for A, B, and C; and Laura Miles (Hartnell College, Salinas, CA) for D.

species (Table 1, Fig. 2). Mitochondrially encoded loci such as the cytochrome C oxidase (*cox*) one and two gene have been useful as well (Choi et al. 2005a; Farahani-Kofoet et al. 2012; Gent et al. 2009b; Klosterman et al. 2014; Summers et al. 2015a). Although both the rDNA and mitochondrial genomes are present in multiple copy number per cell, due to different rates of evolutionary divergence,

mitochondrial loci may be better suited for development of diagnostic markers for closely related species. For example, an ITS-based detection assay for the spinach downy mildew *P. effusa* could not differentiate from the closely related sugar beet pathogen *P. schachtii* (Klosterman et al. 2014), whereas a mitochondrial based assay has greater sequence divergence in the target locus (S. Kunjeti, F. N.

Table 1. Primers utilized for detecting pathogenic downy mildew species using PCR-based diagnostics focusing on primer and probe sequences (if applicable) as well as the target locus utilized for the diagnostic tests

Pathogen species	Plant host	Type of assay	Target locus	Primers/Probes (5'-3')	References
<i>Bremia lactucae</i>	Lettuce	Conventional PCR	Mitochondrial <i>orf286</i>	1F GGAGTCATTGTTTGATTTAACT	Kunjedi et al. 2016
		TaqMan qPCR	Mitochondrial <i>orf286</i>	95R AACTCAGAGCTAGATTACCA F17 aataaatcataaGTCATTGTTTGATTTAACT	Kunjedi et al. 2016
		Conventional PCR	ITS2	R18 aataaatcataaGAGCTAGATTACCA Bl_probe [FAM] ATCAATAGAATGTCCCACTGCAAT [BHQ1]	Mota et al. 2011
<i>Peronospora arborescens</i>	Opium poppy	Conventional PCR	ITS2	Br-fwd GCTCAAAAATCATGGTGTACTG Br-rev GACTGGCTACGAATGACTTCC	Landa et al. 2007
		Conventional PCR	ITS1/ITS2	P3Pa1fw GCTATGGCGATAATGGAGGA P3Pa1rv CAAATTTCCCAAATGGGTG	Landa et al. 2007
		Conventional PCR, Nested PCR and SYBR qPCR	5.8S/ITS2	OMPac1fw CCACACCTAAAACTTTCC OMPac1rv AACACTCCTCCATTATCG OMPac7fw GAACGCATATTGCACTTCC	Landa et al. 2007; Montes-Borrego et al. 2011
<i>Peronospora belbahrii</i>	Sweet basil, sage	Conventional PCR and SYBR qPCR	ITS1	OMPac7rv CGCACAACACAAATTTCC Bas-F CCGTATCAACCCAATAATTTGGGGTTAAT	Belbahri et al. 2005
<i>Peronospora effusa</i>	Spinach	TaqMan qPCR	ITS1/ITS2	Bas-R TTACAATCGTAGCTACTTGTTCAGACAAAG PfsITS-F GTTCGATTTCGGGTATGATT PfsITS-R TCACACAGCAAAGCCAATT PfsITS-probe [FAM/CY5] CGTTCTTCCTGCTGTGGCGG [BHQ1/BHQ2]	Feng et al. 2014b
		TaqMan qPCR	ITS1/ITS2	P1EF GCACCTACCGATTGAATGACT	Klosterman et al. 2014
<i>Peronospora lamii</i>	<i>Salvia</i> spp.	Conventional PCR and SYBR qPCR	ITS1/ITS2	PeR1 CGCTCGCAATTACTTCCAG Pef1 [FAM] ACTGTGACCCCGCT-MGB-NFQ	Belbahri et al. 2005
<i>Peronospora schachtii</i>	Beet	TaqMan qPCR	ITS1/ITS2	LAM-F CACGTGAACCGTATCAAACACTTT LAM-R CGCCATGATAGGGCTTTCTCAA P1BF GCACCTACCGATTGAATGACA PbR1 TCCGCTCGCAATTACTCCAAA Pbe1 [VIC] ACTGTGACCCCGTT-MGB-NFQ	Klosterman et al. 2014
<i>Peronospora sparsa</i>	Boysenberry, strawberry	Conventional PCR and SYBR qPCR	ITS1	P1 CAC GTGAACCGTATCAACC P2 GATAGGGCTTGCCAGTA	Hukkanen et al. 2006
<i>Peronospora tabacina</i>	Tobacco	Conventional PCR	Designed from microsatellites	Pt_Co_for CGACCCACATGGACACATGC Pt_Co_rev CGTAGCTTGCCACAGTGCC	Zipper et al. 2009
<i>Peronospora variabilis</i>	Quinoa	Conventional PCR	ITS1/ITS2	PV6F GTTGCTGGTTGTGAAGGCTG PV6R ATGCTACGCAACCGAAGTCA	Testen et al. 2014
<i>Pseudoperonospora humuli</i>	Hops	Conventional PCR	ITS1/ITS2	HDM07 AGAATTGACTGCGAGTCC HDM04 AGCCACACAACACATAGT	Gent et al. 2009a
		TaqMan qPCR-HRMC	<i>cox2</i>	RT33F AACTCCCCTTATGGAAGGTATT	Summers et al. 2015a
<i>Pseudoperonospora cubensis</i>	Cucurbits	TaqMan qPCR-HRMC	<i>cox2</i>	RT182R CCATGTACAACAGTAGCTGGA HUMprobeSNP105 [FAM] AACAAACAAATACTG [BHQ1] RT33F AACTCCCCTTATGGAAGGTATT RT182R CCATGTACAACAGTAGCTGGA CUBprobeSNP105 [HEX] AACAAACAAATACTG [BHQ1]	Summers et al. 2015a
<i>Plasmopara halstedii</i>	Sunflower	Conventional PCR	28S rDNA	PHAL-F TATCTCTAAGTTGCTTATAC PHAL-R AGCATATACAGCACATACG	Ioos et al. 2007
		TaqMan qPCR	28S rDNA	qPHAL-F TTCCAGTGCTATAATCCGTGGT qPHAL-R GCACATACGCCGAGCGTA qPHAL-P [FAM]-TCGGCGAGCGTGTGCGTGT-[BHQ1]	Ioos et al. 2012
<i>Plasmopara viticola</i>	Grape	Conventional PCR	<i>cox2</i>	Pv-cox2F CAAGATCCAGCAACTCCAGTTATGGA Pv-cox2R ACATTGTCCATAAAAAACACCTTCTC	Kong et al. 2016
<i>Peronospora</i> spp.	n/a	Conventional PCR	ITS1/ITS2	P3Pa2fw TGATCTCGGTCCGAGCTAGT P3Pa2rv TCACCAGTTATACCGCCACA	Landa et al. 2007
	Spinach	Conventional PCR	18S/ITS1/5.8S/ITS2	UF1 TGAATGCGCATCGTGC	Klosterman et al. 2014
	n/a	Conventional PCR	<i>cox2</i>	UR2 AGATGCCACACAACCGAAG Peronosporomycete forward GGCAAATGGGTTTTCAAGATCC Peronosporomycete reverse CCATGATTAATACCACAAATTTCACTAC	Hudspeth et al. 2000

Martin, and S. Klosterman, unpublished). Likewise, almost identical ITS regions among the sister species *P. cubensis* and *P. humuli* may make it difficult to develop species-specific detection assays for these taxa.

Mitochondrial loci have been successfully used to design species-specific identification and detection markers for multiple downy mildew pathogens, including *P. humuli* (Summers et al. 2015a), *Bremia lactucae* (Kunjeti et al. 2016), and *P. cubensis* (F. N. Martin and L. M. Quesada-Ocampo, unpublished), with assay development for *P. effusa* in progress (S. Kunjeti, F. N. Martin, and S. Klosterman, unpublished).

The recent advent of novel sequencing technologies like next-generation sequencing (NGS) together with comparative genomics analyses has led to the discovery of novel and unique genes or genetic regions in multiple downy mildew pathogens (Kunjeti et al. 2016; Withers et al. 2016). From NGS data, de novo assembly of genome and transcriptome of non-model organisms that can be used for the discovery of novel and unique genomic regions and tested for suitability as detection markers has now become quite affordable. Once identified, any such unique genomic markers can be verified for the presence or absence in multiple isolates of the same species through low depth survey of high-copy target genes (Straub et al. 2011) or high-depth sequencing for unique genome fractions (Withers et al. 2016). These technologies have effectively reduced the need for “finished” genomic resources for the development of molecular detection markers. Recently, identification of unique nuclear genomic markers for pathogen detection through use of NGS data and bioinformatics tools has been reported for the cucurbit downy mildew pathogen *P. cubensis* (Withers et al. 2016) and using a similar approach, unique nuclear genomic markers are currently underway for its sister species, the hop downy mildew pathogen *P. humuli* (A. Rahman et al., unpublished). Additionally, using comparative genomics data, unique species-specific mitochondrial sequences were identified in *B. lactucae* that have been used for development of a highly specific TaqMan assay capable of detecting a single sporangium with a C_t of 32 to 34 (Kunjeti et al. 2016). In the future, use of NGS technologies in combination with third generation sequencing (TGS) is expected to greatly improve marker identification capabilities for other downy mildew pathogens as well as improve currently available methods.

Platforms Used for Downy Mildew Diagnostics

Many detection technologies for downy mildews and other oomycetes rely on platforms such as polymerase chain reaction (PCR).

Conventional PCR and Sanger sequencing are reliable methods for diagnostics, but these methods involve substantial time to carry out in the lab. Currently, real-time PCR (SYBR or TaqMan) assays are considered the gold standard for detection and take significantly less sample processing time than traditional PCR methods. One drawback to SYBR green assays is that they typically do not have the same level of specificity as TaqMan and cannot be multiplexed, which prevents the use of internal controls to evaluate amplification efficiency. For real-time techniques, there has been more research in the genera *Phytophthora* and *Pythium* (Martin et al. 2012; Schroeder et al. 2013), with some detection techniques available for the downy mildews and very few detection techniques for the Albuginomycetidae (Armstrong 2007; Spring et al. 2011; van Mølken et al. 2014). Development and validation of molecular assays for downy mildews is complicated by the lack of available genetic material from a wide range of species, minimal sequence data from phylogenetically informative loci to use as potential targets for diagnostic markers, and a yet to be fully resolved phylogenetic status within different downy mildew genera.

Newly developed isothermal DNA amplification technologies offer an alternative to conventional PCR methods for rapid downy mildew detection, thereby facilitating direct, in-field diagnostic capabilities. Although there are different types of isothermal amplification available (Craw and Balachandran 2012), three techniques seem to be the most promising and effective: 1) helicase dependent amplification (HDA), 2) loop mediated isothermal amplification (LAMP), and 3) recombinase polymerase amplification (RPA). During HDA (Biohelix Corporation, Beverly, MA), the DNA template is separated by helicase, which is covered by single stranded DNA binding proteins. DNA polymerase further facilitates the hybridization of two primers to the DNA template. This biotechnology has been used primarily for foodborne pathogens and more recently has been used to detect *Phytophthora* species (Schwenkbier et al. 2015), and holds promise for downy mildew detection.

LAMP is a rapid molecular detection technique that works well on samples that have not been highly purified (e.g., roots) and can deal with samples with mixed alleles. LAMP is known to be tolerant of PCR inhibitors: after plant tissue is ground in buffer it can be directly assayed. The LAMP reaction works by using four to eight primers, which react with the template to create hairpin loops of DNA, often forming significant quantities (Fig. 3A). The reaction is held at 60 to 65°C and after 15 to 60 min, a positive reaction can be read visually

Fig. 2. UPGMA alignment tree of the internal transcribed spacer regions for several economically important downy mildew species. Dark lines denote single nucleotide polymorphisms that are different from the consensus sequence. Furthermore, directional arrows denote species-specific primers that have been developed for each species. Note the reverse primer for *Bremia lactucae* from Mota et al. (2011) is not shown in this image because it is farther into the ITS2 region.

by assessing turbidity or fluorometrically by incorporating intercalating dyes. There are several advantages to LAMP in that software is currently available to develop primers (<http://primerexplorer.jp/elamp4.0.0/index.html>) and resources are available to troubleshoot assay development. The primary disadvantage is that the chemistry is quite different from PCR and it does take significant optimization to get successful and specific amplification (Craw and Balachandran 2012). Additionally, limited information is available about multiplexing reactions and whether it is possible to use this technology for detection of specific SNPs. Thiessen et al. (2016) developed a system to detect powdery mildew (*Erysiphe necator*) of grapes using spore traps with real-time PCR and LAMP to quantify the pathogen in an effort to optimize the timing of fungicide application for improved disease management. Finally, LAMP is by far the most widely published isothermal detection for oomycetes and assays have been developed for *Phytophthora kernoviae* (Tomlinson et al. 2010), *Phytophthora melonis* (Chen et al. 2013), *Phytophthora nicotianae* (Li et al. 2014), *Phytophthora sojae*, *Phytophthora ramorum* (Tomlinson et al. 2010), *Pythium aphanidermatum* (Fukuta et al. 2013), *Pythium hellicoides* (Takahashi et al. 2014), *Pythium myriotylum* (Fukuta et al. 2014), and most recently for *Plasmopara viticola* (Kong et al. 2016).

A newer form of isothermal amplification is recombinant polymerase amplification (RPA), which utilizes several enzymes in a single reaction, an exonuclease, single stranded DNA binding proteins, a polymerase, and recombinases in order to create successful amplification (Fig. 3B). The reaction is incubated at 37°C and successful amplification occurs after 5 to 30 min depending on the type of RPA technology and titer of the target. These enzymes are commercially available in lyophilized pellets from TwistDx (Cambridge, U.K.) and Agdia Inc. (Elkhart, IN). Both manufacturers offer various research kits where some reactions can be read by gel electrophoresis, fluorometric probes, or lateral flow devices. RPA has many advantages; like LAMP, it is tolerant of inhibitors found in plant tissue so DNA extraction is not necessary, but unlike LAMP, primer and probe design is similar to PCR, making it easier to adapt PCR diagnostic assays to this technology. In addition, multiplexed amplifications can be run. An RPA diagnostic assay for *Phytophthora* (which used the same mitochondrial targets as a recent TaqMan diagnostic assay [Bilodeau et al. 2014]) showed specificity when multiplexed with a plant internal control, thus confirming the suitability of the template for amplification (Miles et al. 2015). In separate reactions, there were species-specific detection capabilities for *P. kernoviae*, *P. ramorum*, *P. sansomeana*, and *P. sojae* (Miles et al. 2015; Rojas et al. 2017). These new diagnostic markers have utility in rapidly detecting *Phytophthora* spp. in the field with portable data collection units and should have applications in small diagnostic laboratories, research, and regulatory settings. The ability to sequence the amplicons also provides the ability to confirm species identification and be used in metagenomic studies. In the future, it should be possible to use RPA to delineate important SNPs to identify a particular genotype (e.g., fungicide-resistant isolates), and identify specific clonal lineages of downy mildews.

Downy Mildew Races and Pathotype Markers

Downy mildew pathogens have developed strong relationships with their host due to their obligate nature (Thines 2014). Isolates within a species of downy mildew will frequently have differences in virulence and/or pathogenicity, which have in some cases been resolved by designating pathogen races, pathotypes, or even new species (Gascuel et al. 2016a; Rivera et al. 2016). Race designation has been useful to catalog a virulence or pathogenicity phenotype, but also to classify resistance loci in host varieties for breeding or crop production purposes. That is, races of a downy mildew pathogen will be able to cause disease only on some varieties of the host species in question but not others depending on the resistance loci content of that variety. Spinach downy mildew caused by *Peronospora effusa* (formerly known as *Peronospora farinosa* f. sp. *spinaciae* but now known to be a distinct species), is the most economically important spinach disease and new races of the pathogen have appeared in the

US in the last 15 years (Choi et al. 2007; Feng et al. 2014a). A recent study by Feng et al. (2014a) identified four new races (11, 12, 13, and 14) based on isolate virulence in spinach differentials containing six resistance loci (RPF1 to RPF6). Previously identified races (1 to 10) were not able to infect spinach cultivars with the resistance locus RPF2; nonetheless, the new races 11, 12, 13, and 14 could overcome this locus (Feng et al. 2014a).

Race information for crop production is particularly important if a downy mildew pathogen has a differential spatial distribution due to its soilborne nature or to population structuring due to host availability in a region. Understanding the races of a downy mildew pathogen in a region can be valuable when deploying resistant varieties if they are available. The sunflower downy mildew, *Plasmopara halstedii*, has great economic impact on sunflower crops, also has races identified with virulence to different sunflower cultivars, and can form overwintering oospores that persist in the soil (Gascuel et al. 2015). To date, 36 pathotypes of *P. halstedii* have been identified worldwide and the pathogen can infect several Asteraceae species including *Helianthus* spp., *Bidens* spp., *Artemisia* spp., and *Xanthium* species. The identified pathotypes have stratified spatial distribution due to the soilborne nature of the oospores and differences in host availability in different regions where the disease occurs; thus, local race information has been key to deploy the available resistance genes in commercial sunflower cultivars (Gascuel et al. 2015). While race and pathotype information has been a useful way to classify downy mildew isolates, it is also a cumbersome task due to the need to maintain isolates and test them in planta because of the obligate nature of these pathogens. Also, variability in assays and availability of true to type differential sets of hosts for phenotyping are a challenge of this approach since not all hosts are clonal or near homozygous to ensure homogeneous genetics. Thus, developing markers for races and pathotypes that can substitute for the complex phenotyping assays required for downy mildew isolate classification, is desirable.

With advances in genomics and their applications to downy mildew pathogens, it is becoming clear that the underlying reason for what scientists perceive as races and pathotypes are in part due to differences in the effector repertoire among isolates of a species. Several downy mildew genomes of variable finished quality have been

Fig. 3. Schematic of important aspects of two different isothermal amplification methods discussed in this review. **A**, Primer design elements associated with loop mediated isothermal amplification (LAMP). **B**, Diagram of important elements of the fluorometric form of recombinase polymerase amplification (RPA) including a polymerase, recombinase/primer filaments, single stranded DNA binding proteins, a specialized probe, and an exonuclease.

released to date including *Hyaloperonospora arabidopsidis* (Baxter et al. 2010), *Plasmopara halstedii* (Sharma et al. 2015), *Peronospora tabacina* (Derevnina et al. 2015), *Pseudoperonospora cubensis* (Savory et al. 2012), and *Plasmopara viticola* (Dussert et al. 2016), which have allowed annotation of effector genes in most cases. Several effectors have been linked to specific virulence functions such as the *Arabidopsis thaliana* recognized (ATR) effectors ATR1 (Rehmany et al. 2005), ATR13 (Rentel et al. 2008), ATR5 (Bailey et al. 2011), and ATR39 (Goritschnig et al. 2012) from *Hyaloperonospora arabidopsidis*, the *Arabidopsis* downy mildew pathogen. Fewer studies have focused on linking races and pathotypes to specific effectors. However, a recent study used transcriptome data to identify 54 RXLR and Crinkler effectors, proteins typically involved in host-pathogen interactions during downy mildew infection (Gascuel et al. 2016b). They later analyzed the genetic diversity of these effectors in a set of 35 isolates that had been previously assigned to 14 pathotypes via traditional phenotyping. Interestingly, they developed 8 KASP (Competitive allele specific PCR) markers capable of classifying isolates into 11 of the known *P. halstedii* pathotypes (Gascuel et al. 2016a). This strategy can likely be applied to any downy mildew pathogen with a well-established pathotype system such as *P. effusa* (Feng et al. 2014a) and *Pseudoperonospora cubensis*, the causal agent of cucurbit downy mildew (Thomas et al. 1987); but the large majority of economically important downy mildew pathogens do not have race information or genomic data available yet to make such analysis possible.

In addition, even though effectors are sometimes thought to be a genetic basis for differences in virulence and pathogenicity among isolates, due to the strong selection pressure that host adaptation has on an obligate pathogen such as downy mildew, signatures of host adaptation can be detected in genomic regions not directly involved in pathogenicity or virulence. Two host-specialized clades have been reported in *P. cubensis* (Runge et al. 2011) that have been confirmed by numerous population studies (Kitner et al. 2015; Quesada-Ocampo et al. 2012; Summers et al. 2015b). While both clades are capable of infecting different cucurbit hosts, clade 2 isolates occur more frequently in cucumber, and thus have been suggested to have caused the 2004 cucurbit downy mildew reemergence in the U.S. (Runge et al. 2011). Gene- and microsatellite-based markers have been developed for phylogenetic and population studies that can differentiate the two *P. cubensis* clades, but they can be cumbersome to use as diagnostic markers since that was not their intended purpose when developed (Wallace and Quesada-Ocampo 2017). However, a species-specific nuclear marker selected from a coding region was identified in *P. cubensis* (c2555.3e7) by Withers et al. (2016) that was correlated with the host adaptation observed in clade 1 and clade 2 *P. cubensis* isolates; therefore, this marker can provide information regarding crop risk to particular isolates. Similarly, Rivera et al. (2016) reported host specialization in isolates of *P. halstedii* in the U.S. In 2004, *P. halstedii* caused severe epidemics of downy mildew on rudbeckia, a common landscape ornamental plant. Analysis of isolates of *P. halstedii* from sunflower and rudbeckia using microsatellites revealed that isolates are specialized in these two hosts even though they can infect both hosts in a limited way (Rivera et al. 2016). Nonetheless, diagnostic markers for the two host-specialized clades of *P. halstedii* in sunflower and rudbeckia have not been developed. These examples in *P. cubensis* and *P. halstedii* illustrate that molecular diagnostics can be used to detect races and pathotypes either by directly detecting genomic regions involved in virulence and pathogenicity, such as effectors, or by analyzing markers linked to the phenotype, such as microsatellites.

Interestingly, in some cases, race or pathotype markers can also be linked to other phenotypes such as mating type. In the case of *P. cubensis*, the host-specialized clades reported by Runge et al. (2011) have been found to also have an association with mating type. Cohen et al. (2013) found that isolates of A1 mating type are more frequently found on cucumber and cantaloupe, while A2 isolates occur more frequently on pumpkin, butternut squash, and squash. Thus, the species-specific nuclear marker identified by Withers et al. (2016) in *P. cubensis* provides information regarding crop risk and may

provide some insight to mating type due to the allelic variation it presents according to *P. cubensis* host-specialized clades. Furthermore, signatures of host adaptation in *P. cubensis* have also been identified through comparative genomics analysis of mitochondrial genomes in *Pseudoperonospora* (Martin and Quesada-Ocampo, unpublished) that could offer additional marker sources for crop risk and mating type.

Last, resolving the race and pathotype structure of a downy mildew pathogen by using molecular tools will ultimately aid in unveiling the taxonomy of these pathogens, which is tightly linked to their host specificity due to adaptation processes that have resulted in speciation. *P. cubensis* and *P. humuli* are two sister species with a complex taxonomic history. *P. cubensis* infects plants in the Cucurbitaceae, while *P. humuli* is very host specific to hop. The two species cannot readily be differentiated by morphology or ITS sequence; thus, Choi et al. (2005) suggested that *P. cubensis* and *P. humuli* are a single species. Moreover, a study by Mitchell et al. (2011) determined low infectivity of *P. cubensis* isolates on hop in a lab setting and limited infectivity of *P. humuli* isolates on cucurbits. Experiments by Runge and Thines (2012), also in a lab setting, determined that *P. cubensis* infection and sporulation on hop with limited success, while *P. humuli* was able to infect and asexually reproduce on *Cucumis sativus*. Nonetheless, multilocus and whole genome sequencing approaches clearly show that these are two distinct species (Mitchell et al. 2011; Runge and Thines 2012; Summers et al. 2015b). Species-specific diagnostics for *P. cubensis* have been developed (Withers et al. 2016). Development of species-specific diagnostic markers for *P. humuli* are underway targeting nuclear (A. Rahman and L. Quesada-Ocampo, unpublished) and mitochondrial loci (F. N. Martin and L. Quesada-Ocampo, unpublished). Expanding genomic resources and characterizing populations will be key to resolving the taxonomy of downy mildew pathogens.

Detection and Quantification of Downy Mildew Pathogens from Different Environments and Inoculum Sources

Downy mildews can be quantified from the air, soil, or from the seeds of host plants. Spore traps are commonly used to detect airborne oomycetes and spore identification and counts can be applied as an early warning system for timing of fungicide applications (Klosterman et al. 2014; Kunjeti et al. 2016; Neufeld et al. 2013) (Fig. 4A). Volumetric traps collect spores by vacuuming the air and spores are impacted onto a greased tape or into a collection vial (Burkard Manufacturing Co. Ltd., U.K.). Other traps, such as Rotorods, possess rods coated with adhesive material, which spin at a standard rate to collect airborne inoculum (TSE Systems, Chesterfield, MO). Spores collected on greased tape are typically visualized/counted under a compound microscope; a major limitation of this is the large amount of time necessary to cut adhesive tapes, stain slides, visually count spores, and the potential for misidentification of sporangia. Nonetheless, researchers and growers have successfully implemented spore traps to detect disease (Kong et al. 2016; Thiessen et al. 2016). In *P. cubensis*, a correlation has been observed between disease severity and airborne spore counts (Granke et al. 2014), and battery powered impaction traps are currently being used to monitor *P. cubensis* inoculum (Rahman et al. 2017) (Fig. 4B). One of the challenges of dealing with airborne samples involves dealing with samples with multiple pathogen races. Molecular diagnostic techniques, such as real-time PCR, can be used in tandem with spore trap techniques (Thiessen et al. 2016) to simplify and improve the accuracy of genus, species, and race-specific pathogen detection in the field.

Few studies have been done to date to quantify downy mildew inoculum from soil and seeds. Improving diagnostic capabilities in planta for early infections as well as detection of contaminated seed or soil would significantly improve downy mildew disease management. In the case of basil downy mildew, soilborne oospores of *P. belbahri* were considered a possible source of inoculum in Israel, but the oospores' ability to cause disease has not been determined (Cohen et al. 2013). Although basil downy mildew is currently

diagnosed by visual means, it is encouraging that some molecular assays based on real-time PCR have been developed for detection of the pathogen on infected tissue and seed, but these assays have yet to be widely validated or adopted (Belbahri et al. 2005). Seedborne inoculum of *P. effusa* has also been recovered from contaminated spinach seed as well as detected with an ITS-based diagnostic assay and is believed to contribute to the distribution of new races of the pathogen in the United States (Kunjeti et al. 2016).

Pathogen detection from soil can be complicated by low pathogen inoculum densities, leading to relatively low amounts of extracted pathogen DNA and the presence of PCR inhibitors (e.g., humic acid or phenolics) impacting amplification efficiency. There is still little known about the best method to remove these contaminants from the extracted DNA, but certain extraction techniques have successfully use paramagnetic beads (Bilodeau et al. 2012) or employ a chemical clarifying agent (flocculation) (Bilodeau and Robideau

2014) to improve the purity of soil-extracted DNA. In an effort to determine if PCR inhibitors were influencing amplification efficiency, Bilodeau et al. (2012) developed an internal control that was added to the master mix of a TaqMan assay for *Verticillium dahliae* that was amplified by the same primer pair as the pathogen but had a unique TaqMan probe. Amplification of the internal control when present alone was compared with amplification in extracted soil DNA; an increase in C_t was indicative of reduction in amplification efficiency. Genus-specific primers for *Phytophthora* have been tailed on the internal control and used in assays for *Phytophthora* spp. (Bilodeau and Robideau 2014), *Bremia lactucae* (S. Kunjeti, F. N. Martin, and S. Kolsterman, unpublished), and should work for downy mildew assays of soilborne inoculum as well. An internal control developed by Haudenschild and Hartman (2011) and used the same way indicated above was effective in a detection assay for *B. lactucae* (Kunjeti et al. 2016). One potential complication of using these internal controls is the possible loss of sensitivity of detection when the pathogen is present at low levels due to competition for amplification; in these cases, if there is no inhibition of amplification efficiency, the samples should be rerun without the adding the internal control. Development of these tools will allow for optimization of DNA extraction procedures and ensuring accurate quantification of the pathogen. For instance, using an internal control, Hukkanen et al. (2006) developed a qPCR assay using SYBR Green with control plasmids for quantification of the downy mildew *Peronospora sparsa* in *Rubus* (Arctic bramble). Up to 37 fg of conidial DNA was present in plant material: 0.2 ppm of *P. sparsa* DNA was found using an ITS region (Hukkanen et al. 2006). For detection of the pathogen in seed material, Montes-Borrego et al. (2011) developed a qPCR protocol using plasmid as internal control for quantification of *Peronospora arborescens* in opium poppy for evaluation of seed stock and symptomless infested plants. The authors detected 10 fg of *P. arborescens* per 40 ng of host DNA without losing accuracy. The results suggest that the quantity of *P. arborescens* DNA in commercial seed stock could be as high as 0.256 mg of pathogen DNA per kg of seed (Montes-Borrego et al. 2011).

Future Prospects

There are many challenges associated with diagnostic techniques in plant pathogenic downy mildews. Generally, they involve three

Fig. 4. Spore trap technologies to detect downy mildew species in the field. **A**, Solar powered impactation trap at a spinach research plot in Salinas, CA, to detect *Peronospora effusa* (photo credit to Steven Klosterman, USDA-ARS, Salinas, CA). **B**, Battery powered impactation trap at a sentinel plot at the Central Crop Research Center, Clayton, NC, to detect *Pseudoperonospora cubensis*.

Fig. 5. Mauve alignment of mitochondrial genomes of two downy mildew species and two *Phytophthora* species. Note common marker systems (*atp9-nad9* and *cox1-cox2*) for *Phytophthora* species are denoted from Miles et al. (2017) and Martin and Tooley (2003). Inverted regions of the mitochondrial genomes are also denoted in this alignment.

areas: 1) choosing the appropriate detection technique, 2) identifying a target locus, and 3) providing adequate technology transfer and training once an assay is developed. For the first, choosing a detection technique can be problematic because each technique has advantages and disadvantages (e.g., cost, reliability, sensitivity, skill level required, portability); also, there are a variety of downstream applications that can be used with various diagnostic techniques (e.g., sequencing). Second, choosing a target locus can be problematic. While loci like the ITS region may have significant reference data sets associated with them, this locus does not allow for differentiation among some closely related oomycete taxa. Moreover, if the locus is to be used for quantification, it is important to make sure there is no variation in copy number among isolates or across species. The third issue of technology transfer is frequently overlooked in the research community and is a problem when assays are transferred to diagnostic laboratories or regulatory agencies. When transferring these technologies, it is essential to use known samples and follow the procedure exactly as developed by the researcher. Additionally, providing support through workshops or “ring trial” experiments where known samples are evaluated blind might also be warranted (Martin et al. 2009).

However, there is hope for accurate and rapid detection of downy mildews. Mitochondrial genomes representing a broad range of downy mildew taxa are being assembled and will be available soon (F. N. Martin, unpublished). These will provide a valuable resource for identifying suitable loci for phylogenetic studies as well as development of diagnostic assays. Additionally, in cases where multiple isolates have been sequenced, it is not uncommon to observe intraspecific polymorphisms that are useful for mitochondrial haplotype classification, thereby providing a cytoplasmic marker that can be useful in population studies. Currently, only two mitochondrial genomes are available in GenBank (*P. cubensis* and *P. tabacina*), but in comparison with other *Phytophthora* genomes, there appear to be many unique regions and significant differences in genome length (Fig. 5).

Novel diagnostic approaches such as next generation sequencing methods can be used to find new genetic loci and species-specific markers via comparative genomics (Pallen et al. 2010; Studholme et al. 2011). As opposed to first generation methods that involved Sanger sequencing, which could be time consuming, next generation methods allow for rapid identification of pathogens from environmental samples (e.g., soil, water, plants) using high throughput technologies (Metzker 2010). These new sequencing methods were used to find candidate diagnostic markers for the cause of cucurbit downy mildew, *P. cubensis*, through comparing next generation data to a close relative, *P. humuli* (Withers et al. 2016). PCR was used against a larger collection of oomycete isolates to further validate the candidate markers for *P. cubensis* for a total of seven markers. Rapid and accurate diagnostics of downy mildews and their inoculum sources are essential for quick implementation of effective disease control strategies. A clear benefit from developing early diagnostics for downy mildew pathogens would be to improve existing alert systems (Ojiambo et al. 2011). Future research looking at a direct measurement of inoculum in air, soil, or planting material provides a resource to explicitly link inoculum sources, weather, and host factors to disease risk and identification of potential hotspot areas to survey for pathogens of concern.

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Dr. Crandall received her Ph.D. in environmental studies in 2016 from the University of California, Santa Cruz, where she focused on fungal ecology and ecosystem-based management of special forest products. She has since been conducting postdoctoral research at California State University, Monterey Bay, in the Plant Pathology Laboratory on microbial communities surrounding *Phytophthora*-infected roots and molecular diagnostic tools related to *Phytophthora* and downy mildew species. Her specialties and interests include aerobiology, metagenomics, microbiology, mycology, and plant physiology in managed and unmanaged environments.

In 2015, Dr. Rahman joined Dr. Lina Quesada's research program as a postdoctoral scholar to study genomics and population genetics of the cucurbit downy mildew pathogen, *Pseudoperonospora cubensis*. He is currently leading the investigation in biosurveillance and development of molecular diagnostic makers for early detection of the pathogen. He received his Ph.D. in Plant Pathology from Pennsylvania State University (PSU) and his doctoral work was focused on chemical and biological control agent mediated induced host resistance in perennial ryegrass against *Magnaporthe oryzae* using molecular, biochemical, and analytical tools. Following completion of his Ph.D. in 2014, Dr. Rahman worked at PDA (Pennsylvania Department of Agriculture) as a postdoctoral fellow to study population biology of *Phytophthora* species, an important pathogen of Christmas trees.

Dr. Quesada is an assistant professor in the Department of Plant Pathology at North Carolina State University. Her research program uses a broad range of tools from field studies to genomics to improve management strategies of cucurbit and sweetpotato diseases. She received her B.Sc. degrees in microbiology and biology from Universidad de Los Andes, Bogota, Colombia. She then joined the Ohio State University and worked on genomics of *Phytophthora infestans*. She received her Ph.D. in plant pathology from Michigan State University (MSU) and her doctoral work focused on the population structure of *Phytophthora capsici*. Later she studied worldwide populations of *Pseudoperonospora cubensis* as a postdoctoral researcher at MSU, and *Fusarium* diseases in maize as a NIFA postdoctoral fellow.

Dr. Martin has been a research plant pathologist with USDA-ARS in Salinas, CA, since 1996. He received his Ph.D. in plant pathology from the University of California, Berkeley, and was with the Plant Pathology Department at the University of Florida for 10 years prior to his current position. His research has focused on the ecology, biology, detection, identification, and phylogeny of the genera *Pythium* and *Phytophthora*.

In 2011, Dr. Bilodeau joined the Canadian Food Inspection Agency (CFIA), Ottawa Plant Laboratory (Fallowfield), as research scientist in the Plant Pathogens Identification Research Lab (PIRL). Dr. Bilodeau graduated from Laval University (Quebec, Canada) with his Ph.D. on "detection and genomics of *Phytophthora ramorum*, causal agent of sudden oak death." He is now an associate professor at Laval University in Department of Biochemistry, Microbiology, and Bioinformatics. In 2008, he moved to USDA-ARS Salinas, California, for postdoctoral research on development of detection and quantification tool for plant pathogenic fungi (*Phytophthora* and *Verticillium*) using DNA detection methods. Today, he conducts research in development of technologies to identify plant pests (fungi-oomycetes) of regulatory significance in agriculture and forestry using real-time PCR, nucleic acid extraction, and genomics/metagenomics.

Dr. Miles is an assistant professor in the School of Natural Sciences at California State University, Monterey Bay. His research program focuses on fungal and oomycete pathogens on specialty crops covering a broad range of topics such as fungal genomics, fungicide resistance, metagenomics, postharvest diseases, and molecular diagnostics of plant pathogens. He received his B.Sc. degree in biology at Western Michigan University in Kalamazoo, MI. He then joined the Small Fruit Pathology Laboratory at Michigan State University and completed a Ph.D. in 2011 on anthracnose fruit rot of blueberry caused by the fungus *Colletotrichum acutatum*. Later, he held postdoctoral positions at the University of Idaho (Aberdeen, ID) and USDA-ARS (Salinas, CA), both focusing on molecular diagnostics of various plant pathogens (primarily *Phytophthora*, *Pythium*, and *Rhizoctonia* spp.).

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