INVESTIGATIONS IN ASEXUAL DEVELOPMENT IN *ERYSIPHE NECATOR*: CHARACTERIZATION OF FACTORS AFFECTING CONIDIATION AND IDENTIFICATION OF GENE SEQUENCES ASSOCIATED WITH DEVELOPMENTAL CHANGES

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INVESTIGATIONS IN ASEXUAL DEVELOPMENT IN *ERYSIPHE NECATOR*: CHARACTERIZATION OF FACTORS AFFECTING CONIDIATION AND IDENTIFICATION OF GENE SEQUENCES ASSOCIATED WITH DEVELOPMENTAL CHANGES

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Erysiphe necator is the causal agent of grapevine powdery mildew, one of the most economically significant diseases of grapes worldwide. Spread of the disease is conspicuously enhanced by the ability by the fungus to produce large numbers of asexual spores. In this study, we investigated molecular and environmental cues that are associated with asexual development to gain a greater appreciation for control of this process in *E. necator*. We found evidence that initiation of conidiation is controlled by a signaling process that is potentially affected by both light and colony density. The signal appears to initiate in the center of colonies and travel outward with the radial growth of the fungus. Colonies placed in complete darkness during the theorized time point for promulgation of this signal failed to initiate conidiation, suggesting a role for light in stimulating asexual development Colony density also showed a significant relationship with timing of sporulation events, with latent period decreasing as initial inoculum density increased.

In order to identify genetic sequences which could play a role in asexual development, cDNA-AFLP analysis was used to identify sequences associated with four time points in spore development: vegetative growth (pre-sporulation), conidiophore initiation, full sporulation and ascocarp maturation. Using this method, 231 differentially expressed sequences were identified and sequenced. The majority of these showed no similarity to sequences available in the NCBI database, with the second largest class showing matches to putative fungal sequences of unknown function.

Sequences involved in metabolism, signaling, transcription, transport and protein fate were differentially regulated across these stages of development.

BIOGRAPHICAL SKETCH

Laura Wakefield was born on May 25, 1977 in Pittsburgh Pennsylvania. Her university career began at Pennsylvania State University in State College, PA in 1995. While at Penn State, she pursued a bachelor's of science in Horticulture, which she successfully obtained in May, 1999. While at Penn State, she additionally pursued research interests in plant breeding and greenhouse management and successfully completed an independent research project on light and the initiation of flowering in Regal Pelargoniums.

Dr. Wakefield moved on to graduate studies in plant breeding and genomics at Texas A&M University in College Station. While there, she directed her master's thesis work on developing genomic libraries for roses and peaches. While in Texas, Dr. Wakefield additionally took part in the creation and maintenance of breeding populations for the rose and peach cultivar development program.

After completing her master's thesis, Dr. Wakefield switched from her previous focus on plant breeding and decided to pursue a doctoral degree in plant pathology. She began her doctoral work in this field at Cornell University in 2003. She joined Dr. Seem's epidemiology lab at the Geneva research station shortly after arriving at Cornell and began her work on asexual development of the grape pathogen, *Erysiphe necator*. After completing this work, Dr. Wakefield continued on to begin studies in the Law at the University of Michigan Law School in Ann Arbor, Michigan. She hopes to use her knowledge of both science and the law to eventually pursue a career in intellectual property or environmental law.

I would like to dedicate this dissertation to my parents, Steven and Linda Wakefield.

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GENERAL

INTRODUCTION

Grapes are amongst the most widely planted fruit crops world wide. In the US alone, grapes are planted on over 380,000 hectares, bringing in over $3.3 billion in sales (USDA-NASS). One of the most significant challenges to grape cultivation is powdery mildew, caused by *Erysiphe necator*. Powdery mildew is the most economically destructive pathogen of grapes worldwide (Pearson and Goheen, 1988). Severe leaf infections can cause early senescence, reduced winter hardiness and reduced shoot growth (Nail and Howell, 2005; Gadoury *et al*, 2001). In fruit, even low levels of infections can have serious impacts on wine quality (Gadoury *et al*, 2007).

Presently, powdery mildew on grapes is managed primarily through the use of fungicides. Growers have enjoyed relative success with this program due to a combination of the development of new pesticides and research which has allowed for more efficient use (Ficke *et al*, 2002; Pearson and Taschenberg, 1980; Erickson and Wilcox, 1997; Gubler *et al*, 1996; Wong and Wilcox, 2002). The recent decreasing availability of new fungicides combined with the pathogen's proven ability to evolve resistance (Wong and Wilcox, 2002) has raised concerns about the long term sustainability of relying purely on fungicides for disease control, however, and new strategies to aid or supplant fungicide use are required.

In epidemics of powdery mildew, the ability of the fungus to create large numbers of conidia is a driving factor. Developing strategies for reducing asexual sporulation should therefore have considerable impact on disease spread. Prior to this research, little was known about asexual reproduction in the powdery mildews. The goal of my thesis work was to begin initial studies on environmental and genetic triggers of sporulation in order to shed some light on control of conidiation in *E. necator* and to identify potential targets for future study.

Control of conidiation in model systems

Although little is known about control of asexual development in the powdery mildews, conidiation and the interplay between asexual development, sexual development and secondary metabolism has received substantial study in model systems, particularly in *Aspergillus nidulans* and *Neurospora crassa*. What follows is a review of relevant literature on what was previously discovered in other filamentous fungi, with some discussion of what this might mean for asexual development in *Erysiphe*.

Environmental Effects on Asexual Spore Formation

Production of conidia is influenced by colony nutrition

Early research into environmental inducers of asexual sporulation in filamentous fungi demonstrated that nutrient stress can stimulate conidition, and it has been hypothesized that this response allows dispersal of fungi to more favorable environments (Adams and Timberlake, 1990). Although sporulation in submerged cultures is typically repressed, submerged cultures of *Aspergillus, Neurospora* and *Penicillium* have initiated asexual sporulation when glucose (Martinelli, 1976) or nitrogen (Morton, 1961; Guignard *et al*, 1984) are limiting. Aerial cultures of *A. nidulans* will similarly commence with sporulation when transferred from complete media to media limiting in either carbon or nitrogen (Skromne *et al*, 1995). Although nutrient starvation is not required for conidiation in aerial cultures, it is thought that nutrient stress reinforces commitment to the conidiation pathway (Adams and Timberlake, 1990). Recent work in G protein signaling in *A. nidulans* has reinforced this hypothesis, with stress responses providing a secondary, parallel signal to the primary sporulation pathway (Casas-Flores *et al*, 2006; Chang *et al,* 2004; Lafon *et al*, 2005; Vienken *et al*, 2005).

Light plays a dual role in initiating and suppressing asexual reproduction

A second theme in asexual development is the effect of light on conidiation. Light can have both a stimulatory and inhibiting role on sporulation, with different wavelengths sometimes producing opposing effects in the same organism (Tan, 1978).

Initial investigations in photomorphogenesis demonstrated that ultra-violet light stimulates conidiation in many fungi, including *Alternaria chyrsanthermia, Helminthosporium oryzae* and *Botrytis cinerea* (Leach, 1964; Honda *et al*, 1968; Tan and Epton, 1973). In organisms where conidiation is induced by ultra-violet light, blue light generally has a reversible repressive effect (Tan, 1978). Blue light, however, acts to promote conidition in *Penicillium* and *Trichoderma* species (Sánchez-Murillo *et al*, 2004; Casas-Flores *et al*, 2006) and also regulates the biological clock that controls conidiation in *Neurospora crassa* (Linden *et al*, 1997).

The developmental responses to light have been thoroughly studied in the model organism for asexual development, *Aspergillus nidulans* (Adams *et al*, 1998). In a series of experiments, Mooney and Yager (1990) elucidated the critical wavelengths and exposure period for stimulation of conidiation. A 15-30 minute exposure to red light within 6 hours after submerged cultures are exposure to air is necessary for sporulation-competent colonies to sporulate. Exposure prior to developmental competence or after the six hour window results in a failure to sporulate, and the stimulation of conidiation by red light can additionally be repressed by exposure to far red light in a manner analogous to blue light repression of UV-induced conidiation. In the dark or after exposure to far red light, the colonies instead proceed through sexual reproduction.

Filamentous fungi use a variety of chemical signals to coordinate asexual development

A number of studies have focused on identifying chemical compounds with effects on conidiation. The best characterized of these are the linoleic acid-derived psi (**p**recocious **s**exual **i**nhibitor) factors from *A. nidulans* (Tsitsigiannis *et al*, 2004). The psi factors were first extracted in a group of metabolites that were over-expressed in a mutant that lacked capacity for either sexual reproduction or conidiation (Butnick *et al*, 1984). This extract was further defined as a mixture of three oxylipins, termed PsiA1α PsiB1α, and PsiC1α (Mazur *et al*, 1991). PsiB1α and PsiC1α promote sexual

reproduction whereas PsiA1α represses it, and the relative proportion of the three determines which developmental pathway will be expressed (Calvo *et al*, 2001; Champe *et al*, 1987).

Although several other oxylipins have proven rolls in coordinating fungal development (Tsitsigiannis and Keller, 2007), none of the other identified compounds with effects on asexual sporulation fall within this class, nor do they bear much structural similarity to each other. In *Penicillium cyclopium*, the diterpenes conidiogenene and conidiogenol promote conidiation (Roncal *et al*, 2002) , whereas in *P. funiculosum*, it is the polyketide sporogen-PF1 that has this effect (Katayama *et al*, 1989). In *Trichoderma* spp., 8-carbon volatile organic compounds (VOCs) induce sporulation in the dark (Nemcovic *et al*, 2008), and in at least one pathosystem plant extracts can stimulate conidiation (Leandro *et al,* 2003).

It is apparent that fungi have evolved diverse methods for producing and responding to sporogenic morphogens, even amongst closely related species. The number of identified compounds remains small, however, and significant work remains to be done to identify morphogens from other systems. In addition, although substantial work has been done in *Aspergillus* linking expression of the psi factors with other sporulation triggers such as light (Tsitsigiannis *et al*, 2004), work in other systems lags behind and questions remain in how signaling through these diverse compounds is connected to more conserved pathways like G-protein signaling.

Genetics of Asexual Development

As discussed above, fungi coordinate multiple sensory inputs in balancing sexual, asexual and vegetative development. In the model species, a large number of genes have been implicated in coordinating these responses and in modulating developmental decisions. The following is a discussion of three major classes of such genes: those involved in photomorphogenesis, G protein signaling, and sporulation-specific transcription factors.

The Genetics of Photoreception

As noted above, light was recognized early as an important trigger of asexual development. Although recent work has pointed to other wavelengths of light important for sporulation, the most significant work has been done on the blue light responses in *Neurospora* and the velvet gene (red and blue light) signaling in *A. nidulans*.

Blue light responses are mediated by the white collar proteins in *Neurospora crassa*

In *Neurospora*, all identified light responses are provoked by blue light, including photoconidiation and the entrainment of the circadian clock that controls sporulation (Linden *et al*, 1997). These responses are controlled by the white collar genes, *wc-1* and *wc-2*. WC-1 is a GATA-like zinc finger transcription factor that contains two PAS domains (named for the proteins it is most commonly associated with and used for protein-protein interaction), a nuclear localization signal and a chromophore binding domain (Corrachano, 2007). The chromophore binding domain attaches to the flavin chromophore FAD and acts as the blue light photoreceptor (He *et al*, 2002). WC-2, also a Zn transcription factor, similarly contains a PAS domain (Linden and Macino, 1997).

The white collar proteins affect sporulation primarily through their roles as transcriptional activators. One of the better characterized of these responses is in control of circadian rhythms. In *Neurospora*, new bands of conidia are produced every 22 hours when colonies are grown in complete darkness. In 12:12 light cycles, however, the clock is stretched to 24 hours, with most sporulation occurring late at night (Liu, 2003). This response is due to the interactions of the white collar proteins and the frequency (*frq*) gene. In the dark, the white collar proteins form a heterodimeric complex and bind the light response element of *frq* (Liu and Bell-Pedersen, 2006). Light induces the white collar complex to activate *frq* transcription and thereby resets the developmental clock (Liu *et al*, 2003). FRQ itself forms a complex that inhibits white collar activity, resulting in a decrease in *frq* expression. The oscillations between white collar activation of *frq*

and its subsequent repression is then responsible for the circadian control of conidiation (Liu and Bell-Pedersen, 2006).

Two homologues of WC-1 and WC-2, BLR-1 and BLR-2, were identified in *Trichoderma atroviride*. BLR-1, like WC-1, acts as the blue light receptor and forms a complex with BLR-2 through their mutual PAS domains. In *T. atroviride*, however, the major role of the white collar homologues is through light-induction of conidiation, rather than in entrainment of a circadian rhythm. It is thought that blue light induces activation of early conidiation genes through the BLR zinc finger domains (Casas-Flores *et al*, 2004). White collar homologues have also been shown to play a role in conidiation in *Fusarium* (Estrada and Avalos, 2008).

Although Mooney and Yager (1990) indicated that red light was the critical wavelength for asexual development in *A. nidulans*, more recent work has shown that blue light can also stimulate conidiation. White collar orthologs, termed *lreA* and *lreB*, were discovered in a screen in *A. nidulans*. These genes share <40% homology with their *Neurospora* counterparts, but contain the same essential chromophore, DNA binding and PAS domains. Interestingly, although blue light stimulates conidiation, the roles of LreA and LreB appear to be promotion of sexual reproduction and repression of asexual development (Purschwitz *et al*, 2008).

The white collar proteins remain to date the best characterized of the blue-light receptors in fungi. In addition, the majority of blue light developmental responses have been shown to be coordinated through white collar and its homologues. Because blue and ultra-violet light have defined role in developmental coordination in other fungi, including *Botrytis* and *Alternaria* spp, it seems likely that these complexes could be playing similar roles in diverse fungi.

The *velvet* complex coordinates diverse light responses in regulating development and secondary metabolism in *A. nidulans*

Unlike *Neurospora*, which can only respond to blue light, *A. nidulans* and related fungi show photomorphogenesis in response to both red and blue light. Mutations in the red-light signaling pathway were recognized early with the discovery of the *veA* mutants. In normal *A. nidulans* development, conidia are produced in the light whereas growth in darkness favors sexual reproduction. The *veA* mutant, however, does not respond to this light switching and instead produces higher numbers of conidia in either environment whereas sexual development is repressed (Käfer, 1965). Later investigations revealed that VeA is not itself a light receptor, nor does it bear any structural similarity to other proteins of known function (Calvo, 2008).

In 2005, Blumenstein *et al* discovered a phytochrome in *A. nidulans*, FphA, that appears to play some role in the asexual/sexual red light switch. Mutations in *fphA* showed some derepression of sexual development under red light, but only when expressed in wild type VeA strains. FphA has been shown to form a nuclear complex with both VeA and the white collar orthologs, LreA and LreB, indicating that both blue and red light responses are mediated through the same complex. The interaction of FphA and VeA is dependent upon the chromophore-binding ability of FphA, suggesting that the formation of the complex is itself light dependent (Purshwitz *et al*, 2008). The presence of VeA in the nucleus is also regulated by both FphA and blue light, with VeA appearing mostly in the cytoplasm in the light but in higher nuclear concentrations in the dark. This suggests that light may be repressing sexual development by impairing nuclear transport of VeA (Bayram *et al*, 2008).

Although VeA was of initial interest because of its role in light-dependent development, it is clear that VeA does more than act as the sexual/asexual switch. In *A. nidulans*, regulation of secondary metabolites, including the aflatoxin precursor sterigmatocystin, is also affected by light. Regulation of secondary metabolic gene

clusters in *Aspergillus* is achieved through the global regulator LaeA (Bok and Keller, 2004). In 2008, Bayram *et al*, showed that function of LaeA in the nucleus is dependent upon the presence of VeA and a second protein, VelB. The three proteins form a complex that supports the function of LaeA and thereby influences the expression of the metabolite gene clusters. In the light, localization of VeA to the nucleus is repressed, preventing the formation of the LaeA/VeA/VelB complex and silencing metabolite expression (Bayram, 2008).

The *velvet* gene has been found in other filamentous fungi, but is absent in yeast. Its roles in other fungi do not map entirely on to its functions in *A. nidulans*, however. In contrast with the result in *A. nidulans,* deletion of *veA* in *A. parasticus* results in reduced conidiation. In both *A. parasiticus* and in *A. flavus*, sclerotial production is blocked in *veA* mutants (reviewed in Calvo, 2008). Sequencing efforts in *N. crassa* additionally revealed a *veA* orthology, *ve-1*. In *Neurospora*, production of sexual structures is light independent, creating questions of how this ortholog may be functioning. Deletions of *ve-1* resulted in shortened aerial hyphae and increased conidiation but decreased development of protoperithecia. As with *veA*, expression of *ve-1* is induced by red light, but its function does not appear to be affected by illumination. Interestingly, complementation of *veA* mutants in *Aspergillus* with *ve-1* results in wild type light responses. It is thought that, while Ve-1 does not respond to the same light cues as VeA, Ve-1 integrates into the same signaling pathways that ultimately switch on sexual reproduction (Bayram *et al*, 2007).

While recent investigations have revealed interesting mechanisms by which VeA and its orthologs may be coordinating developmental and metabolic pathways, its mode of action remains frustratingly elusive. The protein contains a nuclear localization signal and a PEST domain (indicating rapid turnover). An N-terminal domain is also highly conserved across fungal species. The exact function of VeA and its turnover points, if

any, remain undefined, however, and this represents an important avenue for future investigations into the role of VeA (Calvo, 2008).

Beyond red and blue: other light responses in fungi

Although the blue and red responses have received the most significant attention in fungi, other wavelengths of light have identified developmental responses. A member of the green-light activated opsin class of photoreceptors, Nop-1, has been discovered in *N. crassa* and recent work has suggested a role in modulation of conidiation-specific gene expression (Bieszke *et al*, 2007). As fungal sequencing information becomes more available, opsins and other photo-receptors may be found in other fungi that have roles in fungal development.

Heterotrimeric G proteins serve as key developmental regulators

Heterotrimeric G protein signaling has been implicated in developmental control across a wide swath of organisms, including fungi, plants, humans and insects (Li *et al*, 2007). In fungi, their roles include cell growth, mating, cell-cell fusion, morphogenesis, virulence, and secondary metabolism. In asexual development, they act in coordinating multiple sensory inputs and in switching between alternative vegetative, asexual and sexual growth patterns (Yu, 2006).

The basic components of G protein signaling are a G protein-coupled receptor (GPCR), a G protein containing an α, β, and γ subunit, and a number of regulators of G protein signaling (RGS) (Yu, 2006). The GPCRs are membrane bound proteins that contain characteristic seven-transmembrane domains. Their localization in the membrane allows for them to respond to outside signals like carbon source, nitrogen levels or certain classes of light. To date, sequence analysis in *Aspergillus* spp. has allowed for the identification of 16 GPCRs, several of which have roles in reproduction (Lafon *et al*, 2006). Two, GprA and GprB, bear similarity to yeast pheromone receptors and play a role in self-fertility (Seo *et al*, 2004). Deletion of a third GPCR gene, *gprD*, leads to promotion of sexual reproduction and reduction in vegetative growth and asexual

sporulation, indicating it may act as a repressor of sexual cycles (Fischer and Kües, 2006).

After binding to a ligand, the GPCRs transmit the signal through their associated heterotrimeric G proteins. In *Aspergillus*, three α units have been identified, FadA, GanB and GanA. FadA was discovered in a series of investigations into fluffy mutants that grow in a purely vegetative state followed by autolysis (Yu *et al*, 1996). Dominant *fadA* mutants are constitutively active, resulting in suppressed asexual and sexual development as well as in down regulation of toxin production (Yu *et al*, 1999). An alternative, interfering mutation conversely resulted in FadA inactivity, with a resultant phenotype of high levels of conidiation (Hicks *et al*, 1997). The opposing results of these negative and dominant mutations led to the conclusion that FadA must act to promote vegetative growth while simultaneously repressing reproduction and secondary metabolism.

While FadA appears to play the most important role in controlling development, recent investigations into a second α subunit, GanB, showed a candidate pathway by which nutrient stress signaling may be integrated into developmental decisions (Brodhagen and Keller, 2006). GanB regulates conidial germination in the presence of a carbon source. Interfering mutations of *ganB* also result in conidiation in submerged cultures, thus suggesting the wild type gene may suppress asexual reproduction. It is possible that GanB may play a role in asexual regulation through sensing of carbon sources (Chang *et al*, 2004). At this point, little work has been done in the third subunit, GanA, and it is unknown whether it also has some effect on development.

Only one β subunit, SfaD, has been discovered in *Aspergillus* spp. Negative mutations of *sfaD* resulted in reduced vegetative growth and hyper-sporulation (Rosèn *et al*, 1999). Loss of SfaD function does not suppress the dominant active *fadA* mutation, however, indicating that FadA must not rely on SfaD for signaling (Yu, 2006). It is believed that SfaD acts as a heterodimer with the γ subunit, GpgA. Unlike with negative

mutations in *fadA* and *sfa*D, negative mutations in *gpgA* result in reduced conidiation. The combination of both SfaD and GpgA is necessary for ascocarp initiation and development in outcrosses, indicating a role for the heterodimer in sexual reproduction (Seo *et al*, 2005).

G proteins transmit their signals via three pathways in fungi: adenylyl cyclase-cAmp dependent protein kinase A (PKA) 2) mitogen activated protein (MAP) kinase pathways and 3) IP_3-[Ca^{++}]-DAG (diacyl glycerol) dependent protein kinase C (Yu, 2006). Of these, the first two have been shown in sporulation associated pathways. FadA has been theorized to use PKA to transmit its signal (Shimizu and Keller 2001). Although not linked to a specific α subunit, members of the MAP kinase family also have demonstrated effects on sporulation. The MAP kinase MpkA has roles in hyphal morphogenesis and others, such as SakA, are upregulated in early asexual sporulation events (Fischer and Kües, 2006).

The signaling processes of the GPCRs and their associated G proteins are modulated by the regulators of G protein signaling or RGSs. RGS proteins contain a 130 amino acid domain that interacts with Gα subunits to turn off GPCR mediated signaling (Yu, 2006). Several RGSs have been identified in *Aspergillus,* with two showing well characterized roles in asexual development. FlbA interacts with FadA to modulate its suppression of asexual and sexual development. Negative mutations in *flbA* show the same phenotype as constitutive expression of *fadA*. Current models hypothesize that FlbA negatively regulates FadA function and allows for sexual and asexual development. (Yu *et al*, 1996). RgsA, a second *Aspergillus* RGS, plays a similar role in attenuating GanB signaling (Chang *et al*, 2004).

Although not a member of the classic G protein pathway, the protein FluG has also been shown to play a critical role in G protein signaling in *A. nidulans*. Mutations in *fluG* result in a fluffy phenotype (Adams *et al*, 1992). Negative mutants of *fadA* or *sfaD*, which ordinarily present enhanced conidiation, do not sporulate at all in *fluG* mutants,

suggesting that the asexual sporulation pathway ordinarily suppressed by active FadA depends upon FluG for function. Wild type *fluG* colonies can complement *fluG* null mutants when separated by a membrane, which led to the conclusion that FluG creates a small diffusible signal that is able to cross the membrane and activate sporulation. The identity of this signal has yet to be determined, but it has been hypothesized that it acts in a dose dependent manner, with asexual development being triggered when the concentration exceeds some critical threshold (Adams *et al,* 1998). Interestingly, initiation of sporulation triggered by stress responses is not dependent upon FluG, supporting the idea that nutrient stress responses involve a parallel, reinforcing pathway to the primary asexual initiation genes (Lee and Adams, 1996).

Given their common roles in coordinating development across kingdoms, it is not surprising to find that similar G proteins have functions in other filamentous fungi. Heterotrimeric G proteins have demonstrated roles in the asexual development of many fungi, including *Penicillium*, *Trichoderma*, *Alternaria*, *Cryphonectria* and *Neurospora* (Garcia-Rico *et al*, 2008; Koman-Zelazowska *et al*, 2007; Yamagishi *et al*, 2006; Segers *et al,* 2004; Kays and Borkovich, 2004). Some differences appear in the ultimate signaling pathways used by the various G proteins; FadA in *A. nidulans* is associated with Protein Kinase A/cAMP signaling whereas the corresponding α subunit in *Penicillium*, Pga1, appears to be cAMP independent, for example (Garcia-Rico *et al*, 2008). Despite these variations, however, it seems that the basic model of G protein mediated asexual development is a common strategy amongst the filamentous fungi and further G protein systems may be expected to play a role in other, less characterized systems.

Small G proteins also coordinate development

G proteins of the Rho and Ras families have also been implicated in developmental control in filamentous fungi. These small proteins bind both GDP and GTP and are active when bound to the latter (Fischer and Kües, 2006). In *Aspergillus,*

Ras has been shown to be necessary for germination of spores, but conidiation depends on decreasing concentration below a critical threshold (Adams *et al*, 1998). Rho coordinates with Ras in controlling hyphal polarity and development in *Penicillium* (Boyce *et al*, 2005) and is necessary for differential vegetative versus conidial septation in *Neurospora* (Rasmussen and Glass, 2007). Investigations into the roles of small G proteins such as these are complicated by the lethal phenotype of null mutants, but it seems likely that further morphogenesis functions will be found for Ras and Rho and potentially for other small G protein families like Ran and the filamentous-fungi-specific Rac family.

Sporulation transcription factors show complex, interdependent expression in *A. nidulans*.

Although a number of transcription factors have been shown to have some function in asexual fungal development, the most prominent of them are the zinc finger transcription factors (Fischer and Kües, 2006). As discussed above, the white collar proteins WC-1 and WC-2 fall within this family and act in the blue light signaling sporulation pathway in *N. crassa*. Zinc finger proteins have also been implicated in developmental control in *Aspergillus, Magnaporthe*, and *Chryphonectria* (Adams *et al*, 1998; Zhou *et al*, 2009; Sun *et al* 2009).

In *Aspergillus*, one of the earliest discovered transcriptional regulators, BrlA, was identified as a Cys_2-His_2 zinc finger transcription factor. Mutants of *brlA* were so named because these colonies form tall indeterminant conidiophore stalks but no vesicles, giving the colonies a bristle appearance (Adams *et al*, 1998). A series of investigations with bristle mutants proved that BrlA has a role in early development and that it is necessary for spore development (Mirabito *et al*, 1989).

Control of *brlA* expression turned out to be unexpectedly complex. The wild type *brlA* gene contains two overlapping transcription units, *brlA*α and *brlA*β, and expression of both is required for proper spore development (Adams *et al*, 1998). The two reading

frames appear to be independently regulated, with BrlAβ requiring some post-translational modification for full activity as well (Han and Adams, 2001). It is possible that this multi-level control of *brlA* expression allows *Aspergillus* to temper development in response to multiple signals (Adams *et al*, 1998). Recent investigations in sporulation in *A. fumigatus* revealed a role for BrlA in responding to nitrogen stress, so it appears that nutrient starvation may be one environmental cue that is integrated through complex *brlA* expression (Twumasi-Boateng *et al*, 2009).

BrlA is required for expression of a second transcriptional activator, *abaA*. AbaA in turn activates *brlA* expression, leading to a mutual feedback loop. AbaA contains a DNA binding domain and a leucine zipper for possible dimerization. Null mutants of *abaA* mutants fail in middle stages of conidiophore development, producing long strings of cells on each conidiophore that appear like beads on a string or an "abacus". One of the primary functions of AbaA appears to be in regulation of later-stage conidiophore development genes (Adams *et al*, 1998).

Expression of *brlA* is also dependent upon action by FluG and FlbA. Screens for further fluffy mutants also showed other transcriptional regulators which act upstream of BrlA. FlbC and FlbD contain DNA binding domains and are theorized to act as direct regulators of *brlA* expression (Yu *et al*, 2006). The basic zipper protein FlbB is also thought to act as a transcriptional regulator with possible direct effects on *brlA* expression and was recently hypothesized to create a small diffusible conidiation factor (Etxebeste *et al*, 2008).

Sporulation in *Erysiphe necator*

Previous studies in *E. necator* have established a basic model for the timing of asexual sporulation events. Following inoculation, *Erysiphe* colonies grow in a purely vegetative state for a period of 5-9 days. Conidiophores then appear singly and in groups throughout the body of the colony, suggesting the presence of some signal which coordinates asexual development. This pattern of purely vegetative growth followed by

asexual sporulation indicates that *Erysiphe* may have the same need to acquire developmental competence before sporulation in a manner analogous to that observed in *Aspergillus* (Mooney and Yager, 1990). It may also be that *Erysiphe* contains some repressor of asexual and sexual development such as FadA that keeps the colonies in a purely vegetative state.

Unlike *A. nidulans*, *Erysiphe* is bipolar heterothallic and sexual reproduction requires pairing of compatible mating types (Gadoury and Pearson, 1991). Observational data suggest that asexual sporulation is repressed upon commencement of mating. When colonies of opposite mating type are inoculated in close proximity on a leaf, a zone of conidiation inhibition appears between the colonies as the hyphae merge, and eventually asexual sporulation is entirely switched off across both colonies. In established colonies in the field, the arrival of a second mating type results in suppression of production of fresh conidia and eventually extant conidiophores shrivel.

The established paradigms for control of asexual sporulation in model systems have some implication for sporulation in *Erysiphe*. As discussed above, the period of vegetative growth before induction of conidiation in *Erysiphe* bears some similarity to patterns of growth in *Aspergillus*. In the model systems, several common themes appear in the roles of light, G proteins and zinc finger transcription factors in developmental control. *Erysiphe* shows the same pattern of asexual versus sexual development that is coordinated by these models and it may be that the established schemes may prove a helpful model for *Erysiphe* development.

Erysiphe may be expected to differ from model pathogens in a number of respects, however. It is not as closely related to the model species as they are to each other, and it could well be that entirely different means of control evolved in *Erysiphe*. The powdery mildews as a group are furthermore obligate biotrophs with perhaps greater dependence on host cues for developmental decisions. In addition, the heterothallic

nature of the fungus suggests that the asexual v. sexual paradigm may be under considerably different control than in the homothallic *Aspergillus* spp,

The goal of this work was two fold: first, to investigate environmental triggers such as light in the development of *Erysiphe*, and second, to begin characterization of genes associated with sporulation processes. It was our hope to establish where *Erysiphe* followed established patterns for development and where new, unique systems may govern sporulation processes.

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CHAPTER ONE: INITIATION OF CONIDIATION IN *ERYSIPHE NECATOR* IS REGULATED BY PRIOR VEGETATIVE GROWTH, INOCULUM DENSITY AND LIGHT

Abstract

Initiation of asexual sporulation in powdery mildews is preceded by a period of superficial vegetative growth of mildew colonies. We found evidence of signaling in *Erysiphe necator* that was promulgated at the colony center and stimulated sporulation throughout the colony. Removal of the colony center after putative signal promulgation had no impact upon timing of sporulation by 48-hr-old hyphae at the colony margin. However, removal of the colony center before signaling nearly doubled the latent period. A relationship between inoculum density and latent period was also observed, with latent period decreasing as initial colony density increased. The effect was most obvious in lower inoculum densities, with latent period leveling off beginning with densities of greater than 10 spores/mm^2. Light was furthermore shown to be critical for initiation of sporulation. Colonies receiving light early in colony development but which were submitted to complete darkness 36 hours post inoculation failed to initiate conidiation, but commenced with asexual development once returned to the light.

Introduction

Erisyphe necator, the causal agent of grapevine powdery mildew, is the most economically significant fungal pathogen of grapes worldwide (Bulit and Lafon, 1978; Pearson and Goheen, 1988). Leaf infections can result in reduced vigor, early leaf senescence, reduced winter hardiness and reduced shoot growth in the following season (Nail and Howell, 2005; Gadoury *et al*, 2001). In cluster infections, even low levels of disease can cause degradation in wine quality, increased susceptibility to *Botrytis cinerea* and secondary spoilage organisms, and reduced yield (Gadoury *et al*, 2007)

Control of powdery mildew in commercial fields is achieved primarily through the use of chemical pesticides, with a cost to US growers of over $300 million per year.

Fungicide efficacy has been often threatened by the ability of the fungus to gain resistance to each class of fungicides over time. Beginning with Benlate in the early 1980s (Pearson and Taschenberg, 1980), continuing with the DMIs in the late 80s (Erickson and Wilcox, 1997; Gubler *et al*, 1996), and ending most recently with the strobilurins (Wong and Wilcox, 2002), populations resistant to each class of fungicides have arisen, in some cases only a few years after the introduction of a new class.

Previous investigations into onset of asexual sporulation in *E. necator* have defined the rate-determining relationships between temperature, relative humidity, and latent period, as well as quantitative effects upon numbers of conidia produced and their survival (Delp, 1954; Pearson and Gadoury, 1992; Carroll and Wilcox, 2003). However, the factors controlling the initiation of conidiation itself remain poorly understood. The continual production of vast numbers of conidia is a driving force in epidemics of grape powdery mildew. Thus, disruptions in the timing or volume of conidiation by the pathogen could significantly slow or even stop the progress of disease. Increased understanding of the process of conidiation might allow for direct manipulation of the process, resulting in slowed or reduced production of conidia in the field. Additionally, knowledge of conditions that affect the latent period of the disease or trigger the production of conidia would be of use in the construction of better forecasting models, thus allowing for more precise timing of fungicides. Our objective was to define further triggers and inhibitors of sporulation.

Materials and Methods

Microsurgery experiments. Expanding leaves were detached from mildew-free potted vines of *Vitis vinifera* 'Chardonnay', surface sterilized, and were placed on 1 % water agar in Petri plates. A suspension of conidia of *E. necator* was prepared using distilled water containing 0.005% Tween^{TM} 20, and was adjusted to a concentration of approximately 10^5 conidia per ml. For each experiment, 8-10 leaves were inoculated by dispensing 5 µl droplets of the above suspension onto each side of the midvein using a

digital pipette. The resultant colonies were incubated under 12 hour day/night cycles at 22 C. At 3 and 4 days after inoculation, the edges of four colonies were marked at four points with a fine-point indelible marking pen. Twenty-four hours later, i.e., 4 or 5 days after inoculation, the center of the colony as defined by the area within marked points was excised. Un-cut colonies were reserved as controls. Each day following excisions, the control colonies and the remaining colony margins were observed for signs of sporulation. Each excision time was replicated on four colonies and the experiment was conducted two times.

Effect of Inoculum Density on Latent Period. A series of conidial suspensions ranging from approximately 2-500 conidia/5 µl drop was made. Each suspension contained half the number of spores per 5 ul drop as the previous one, giving a total of eight suspensions of decreasing spore concentration. Grapevine seedlings were grown from seedlings harvested from *V. vinifera* 'Riesling' as previously described (Ficke *et al*, 2003). Two types of mildew-free plant material were inoculated: intact seedlings grown in clear polycarbonate cups and detached seedling leaves on water agar. Each of 10 leaves of each source of plant material received one 5 ul drop of each suspension and suspensions were allowed to dry before placing the leaves 12 hour day-night conditions at 22 C. Percent conidial germination was measured by placing 3 drops of the densest suspension on three glass slides and placing them in a moist chamber at 22 C. After the slides had been incubated for at least 4 hours the percent of germinated conidia determined by counting at least 100 conidia on each of the three slides. The density of inoculation was determined from the area of the spore deposit resulting from the 5 µl droplets (mean of 4.43 mm^2) adjusted for the percentage of germinating conidia within each prepared conidial suspension.

Starting four days after inoculation, colonies were observed twice daily for signs of sporulation. The experiment was repeated four times with each of two clonal isolates of *E. necator* that were isolated from NY vineyards.

Effect of Light on Sporulation. Grapevine seedlings were grown from seed harvested from *V. vinifera* 'Riesling' as previously described. Detached leaves from seedlings were surface sterilized and placed on 1% agar plates. Each leaf was inoculated with five 5 ul drops of a conidial suspension containing approximately 250 conidia per drop. Colonies were allowed to develop under 12 hour day-night cycles at 22 C. Thirty-six hours after inoculation, leaves were divided into three groups: one receiving 24 hours of light per day, one receiving 12 hours of light, and one receiving no light. Starting on day five, sample leaves were permanently removed and observed for signs of sporulation. On day eight, leaves from all three groups were observed for conidiophores/ cm^2 and number of spores produced as measured by counting the spores adhering to a glass slide after it was pressed to the center of each colony. Also on day 8, colonies which had received no light were left in 12 hour day night conditions and observed a second time on day 9. Within each light treatment, a total of 15-20 leaves were sampled and the experiment was conucted a total of four times.

Results

Microsurgery Experiments. Removal of the colony center on day 4 greatly delayed sporulation, but removal of the colony center on day 5 had no effect on the length of the latent period. Excision of the colony center at 4 days post inoculation delayed sporulation in the remaining hyphae until 10 dpi in all replicates and in both repeats of the experiment (Fig. 1). A similar uniformity of response was observed in both control leaves and those whose colony centers were excised on day 5 after inoculation: all sporulated on day 7 following inoculation (Fig. 1). At the time of sporulation on day 7, the sporulating hyphae remaining at the margin of colonies whose centers were excised on day 5 were a maximum of 2 days old.

Density Experiments. The length of the latent period varied from a maximum of 9 days to a minimum of 6 days depending upon the density of inoculation (Fig. 2). Latent period

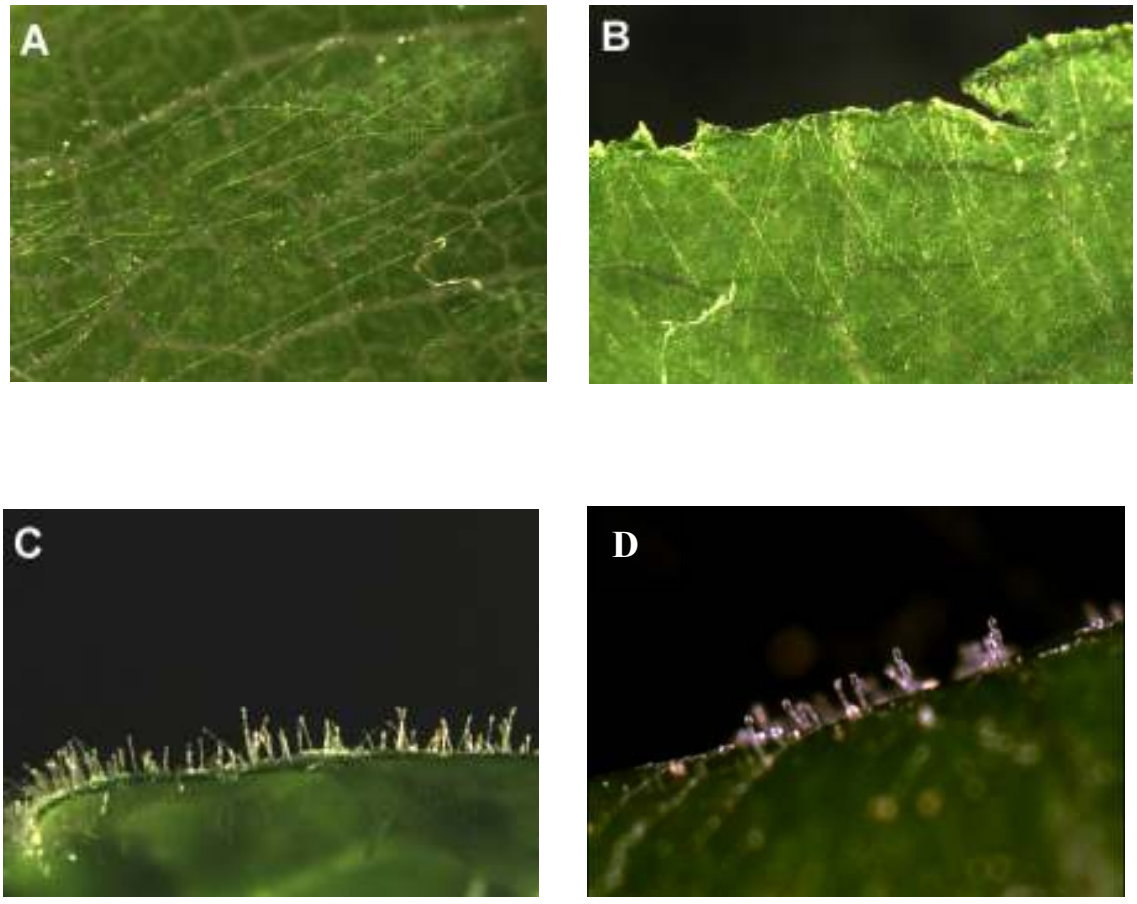

Figure 1. The effect of surgery on powdery mildew colony development on grape leaves. (A) Pre-sporulation growth at margin of 4-day old mildew colony; (B) Continued radial growth of mildew colony without sporulation on day 7 following excision of colony center on day 4; (C) Sporulation at margin of mildew colonies on day 7 following excision of center of mildew colony on day 5. (D)Colonies that were not surgically altered sporulated on day seven

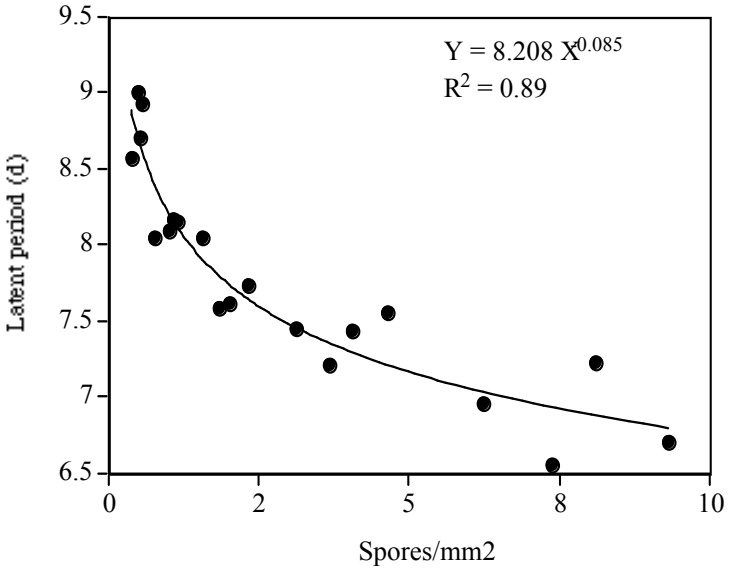

Figure 2. The effect of colony density on latent period. The relationship between the number of germinable conidia per mm^2 and latent period of *Erysiphe necator* on detached leaves of *Vitis vinifera*

decreased exponentially as the number of germinable conidia increased above 1 per mm^2 (Fig. 2) until inoculation density reached approximately 5 to 10 germinable conidia per mm^2 (Fig. 2). Regression of the observed latent period against the number of germinable conidia per mm^2 yielded the following:

$$Y = 8.208\ X^{-0.085}$$

$$R^2 = 0.89$$

where: Y = Latent period (days), and

X = Density of germinable conidia per mm^2.

Increasing the number of germinable conidia above 10 per mm^2 had no significant effect ($P = 0.05$) upon the duration of the latent period.

Effect of Light on Sporulation. In all colonies observed, light was shown to be critical for initiation of sporulation (Fig. 3). While colonies grown in either twelve or 24 hours of light started showing signs of sporulation on day 5 and achieved 100% sporulation by day 7, all colonies kept in the dark after 36 hours post-inoculation owed no signs of sporulation. Colonies that had been placed in the dark but removed on day 7 showed growth of conidiophores at the margin of the colony on day 8 and produced their first mature conidia on day 9, proving that colonies grown in the dark are competent for sporulation but lack some critical trigger. In addition, colonies grown in 24 hours of light showed slightly higher density of conidiophores/cm^2 and numbers of spores produced, although this difference was not significant (data not shown).

DISCUSSION

Previous studies in conidiation in *E. necator* have established that, in a colony deriving from a single mating type, there is a period of vegetative growth for approximately 5-9 days before emergence of upright conidiophores, upon which are born the asexual conidia (Pearson and Gadoury, 1992). This pattern of a period of asexual growth before colonies become competent for sporulation has been established in other model fungi, including *Aspergillus nidulans* and *Neurospora crassa*. (Adams *et al*, 1998;

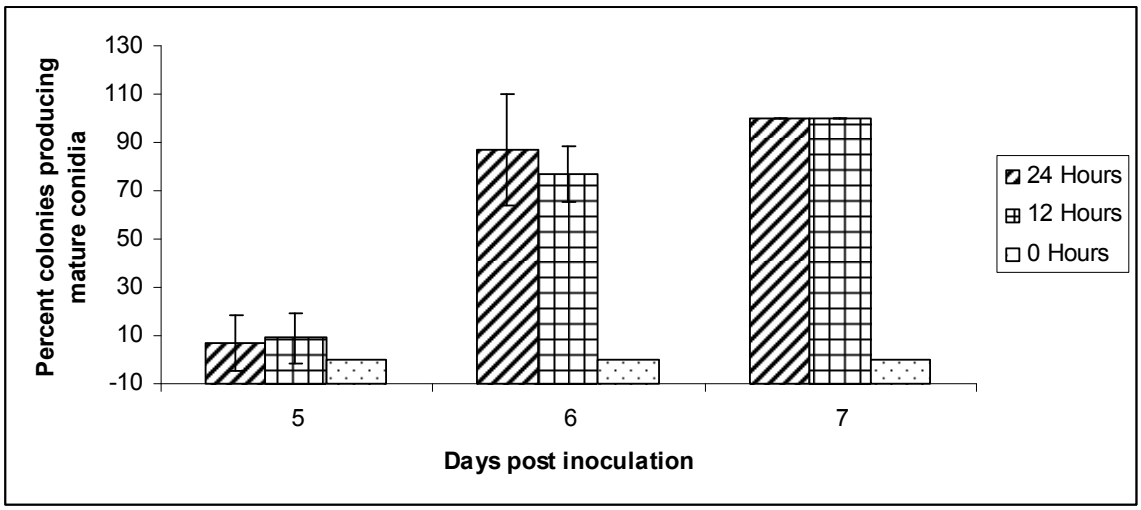

Figure 3: The effect of light on initiation of sporulation. Colonies were inoculated on detached seedling leaves and grown for 3 days on a 12 hour light cycle. At 36 hours post inoculation, the leaves were divided into three groups, 1 receiving 24 hours of light, 1 remaining at the 12 hour light cycle, and 1 receiving zero light. Starting at 5 dpi and continuing each day after , 1/3 of the leaves in each group were destructively sampled and observed for percentage of colonies producing mature conidia.

Bailey-Shrode and Ebbole, 2004). At approximately 6-9 days after inoculation, conidiophores appear singly and in small groups throughout *E. necator* colonies, suggesting a possible signal which coordinates appearance of these structures throughout the body of the colony.

The results from the microsurgery experiments further underscore the idea that the initiation of asexual sporulation is triggered through a coordinating signal. In our experiments, colonies receiving surgery 5 dpi or later were able to sporulate 7 days after inoculation, in time with unaltered controls, while colony margins removed from the main body of the colony on day 4 were not. At the time of sporulation, the oldest tissue in colonies receiving surgery on day 4 was also seven days old. In effect it seems the sporulation clock is reset by removing the colony center on day 4, but by day five, the sporulation process has already been initiated and cannot be slowed by removing colony centers. This suggests that some sort of conidiation initiation signal exists that had either not been sent or not yet reached sufficient strength by day four, but had been fully promulgated throughout the colony, including the one day old margin, by day five.

Two further lines of evidence from the above series of experiments suggest, however, that initiation of sporulation is not purely a function of time. In the experiments linking inoculum density to latent period, a clear relationship between the two was seen, where increasing amounts of inoculum lead to significantly reduced latent periods. This suggests that perhaps colony density, rather than age, is a driving factor in the promulgation of the sporulation signal. Perhaps colonies must achieve a critical density or perhaps some signal produced by the colony must reach some critical level before sporulation can be initiated. This follows what has previously been observed in asexual sporulation studies in *Aspergillus nidulans.* The *fluG* gene in *A. nidulans* produces a small diffusible factor necessary for sporulation, and it has been suggested that sporulation in air is triggered when the concentration of FluG exceeds some necessary threshold (Lee and Adams, 1996).

Although fungal quorum sensing phenomena have not been well characterized, other morphogenesis events in fungi have been similarly linked to density responses. Quorum sensing was first discovered amongst the eukaryotes in *Candida albicans*, where farnesol acts as the quorum sensing molecule that suppresses both the mycelial form of the fungus and the formation of biofilms above a threshold population (Hornby *et al*, 2001; Ramage *et al*, 2002). A similar link between population density and colony morphology has also been observed in the elm pathogen *Ceratocystis ulmi* (Hornby *et al*, 2004) and has been suggested as a general phenomenon in dimorphic fungi (Nickerson *et al*, 2006.

Should quorum sensing play an active role in asexual sporulation in *E. necator*, disrupting the quorum signaling pathway may prove an effective means of control. Disruption of quorum signaling has proven effective in reducing pathogenicity of several gram-negative bacterial pathogens, and transgenic crops expressing disrupters of quorum signaling have shown increased resistance in lab trials (Cui and Harling, 2005). It is possible that similar strategies might be employed in fungi such as *E. necator* that also rely on quorum sensing for critical developmental stages.

These results also have implications for current forecasting models for the development of powdery mildew epidemics in the field. In most field experiments, colonies are produced from high densities of inoculum to ensure a high level of successful infection. This is in contrast to what happens in nature, however, where colonies are more likely started from single or small clumps of spores deposited on leaves by wind or insects. It is possible that the latent period in extant models is underestimated and would need to be adjusted to reflect natural conditions.

The relationship between colony density and conidiation may also explain another phenomenon observed in powdery mildew epidemics—the appearance of diffuse infections on clusters. In *V. vinifera*, the clusters exhibit high levels of susceptibility for a period of about two weeks post bloom, upon which ontogenic resistance develops and

further new infections of powdery mildew are halted. Diffuse infections, which display sparse hyphae and no conidiation, start when the berries are reaching the end of their period of susceptibility. It could be that as the berries achieve full resistance, full development of the colony to a density which would allow for conidiation is halted, thus keeping the colonies in their observed, diffuse, purely vegetative state.

Beyond a possible link between colony density and initiation of sporulation, our studies also established that light is critical for asexual sporulation in *E. necator*. The role of light as an inducer of asexual development is a common theme in filamentous fungi. In *Aspergillus nidulans*, asexual sporulation is repressed in the dark whereas sexual development and mycotoxin production is induced. Both blue and red light can induce reduced sporulation on their own and, when used in concert, produce similar levels of sporulation as white light (Purschwitz *et al*, 2008). Photoinduction of conidiation by blue light has also been demonstrated in the fungi *Paecilomyces fumosoroseus, Alternaria tomato*, and *Trichoderma atroviride* (Sánchez-Murillo *et al*, 2004; Kumagai, 1989; Casas-Flores *et al*, 2006). Several light sensing molecules have been identified in fungi, including a phytochrome in *A. nidulans* that represses sexual development in the presence of red light, FphA (Blumenstein *et al*, 2005), and it is possible similar complexes may be active in *E. necator*.

Due to the obligate nature of *E. necator*, it must be noted that it is impossible to entirely separate the effects of growth in complete darkness on the pathogen from the effects on the plant. It could be that the lack of sporulation observed on colonies grown in the dark could be from an indirect effect from of lack of light on the host rather than a direct effect on the pathogen. We feel this is unlikely for several reasons. First, after removal from the dark, colonies exposed to light exhibited emergence of conidiophores generally within a few hours. If the lack of sporulation in dark-grown colonies was the result of essential nutrients depleted in light-stressed leaves, it seems unlikely that detached leaves placed on nutrient-free water agar could recover quickly enough to

provide that lacking nutrient in just a few hours. Secondly, previous observations on sporulation and plant nutrition in the *E. necator*/*V. vinifera* pathosystem have demonstrated that colonies sporulate more vigorously when excess nitrogen fertilizer is applied to host plants and show reduced sporulation in nutrient starved plants (Gadoury and Pearson, 1988).

In all, the above outlined series of experiments has established that there is evidence for a signal coordinating induction of sporulation in *E. necator* and that this signal may be affected by both colony density and the presence of light. The role of colony density in particular in early developmental events has significant implications both for current forecasting models and for future avenues of control. Further investigations into conidial morphogenesis of young colonies should provide more insight into how the pathogen coordinates development with its environment and its host.

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CHAPTER TWO: cDNA-AFLP ANALYSIS OF SEQUENCES EXPRESSED DURING DEVELOPMENT IN *ERYSIPHE NECATOR*

Abstract

Erysiphe necator is the causal agent of grapevine powdery mildew, one of the most economically significant diseases of grapes worldwide. In this study, we used cDNA-AFLP analysis to identify sequences associated with four time points in spore development: vegetative growth (pre-sporulation), conidiophore initiation, full sporulation, and development of mature ascocarps. Selective amplifications with 45 primer combinations produced approximately 1,600 fragments of which 620 (39%) showed differential expression. We sequenced 231 of these fragments. Forty-five percent showed no homology to identified sequences and an additional 16% showed homology to fungal genes of unknown function, indicating a significant role for uncharacterized genes in developmental programming in this pathogen. Sequences involved in metabolism, signaling, transcription, transport and protein fate were differentially regulated across these stages of development.

Introduction

The pathogen *Erysiphe necator* is the causal agent of grapevine powdery mildew, one of the most significant fungal disease of grapes worldwide. The disease causes severe losses across a wide variety of climates and is responsible for decline in vine health, lower yield, increased susceptibility to other diseases, and lower wine quality (Pearson and Goheen, 1988; Nail and Howell, 2005).

In the powdery mildews, production of asexual conidia is a driving force of epidemics. Previous investigations of latent periods in powdery mildew infection indicated that temperature and humidity can influence the production of asexual spores (Delp, 1956; Pearson and Gadoury, 1992; Carroll and Wilcox, 2003). Recent efforts in our lab established evidence for a signal that coordinates the onset of asexual

reproduction. Further lines of evidence suggest that the production of this signal is influenced by the presence of light and the density of the colony (Chapter One).

At the present time, however, no work has been done to uncover the genetic basis for these signals or how these signals might control powdery mildews in general. Significant work has been done in the model pathogen *Aspergillus nidulans* in establishing the signaling pathways for developmental control (Fischer and Kües, 2006). These studies and related investigations in *Neurospora* and *Penicillium* have revealed roles for light signaling, heterotrimeric G proteins and coordinated production of asexual spores and secondary metabolites (Kasuga and Glass, 2008; Calvo, 2008; Garcia-Rico *et al*, 2008, Yu *et al*, 2006). The implications of these basic models for control of asexual sporulation in *E. necator* are unknown however.

The goal of the present research was to identify sequences differentially expressed during vegetative growth and throughout asexual and sexual reproduction in *E. necator*. In this paper, we used cDNA-AFLP analysis to identify sequences associated with the developmental stages. Our investigations show some overlap between sporulation-associated sequences in *E. necator* and other model systems, but also indicate the fungus may substantially rely on unique genes.

Methods

Plant material, inoculum and pathogen growth

Seedlings were grown from seeds collected from open pollinated Chardonnay and Riesling vines according to Ficke *et al* (2003). The seedlings were grown under mildew-free conditions at constant 23° C in 12 hour photoperiod until the 5-6 leaf stage under mildew-free conditions. Two isolates of *E. necator*, 10-18 and 10-36, of opposite mating type were collected from New York vineyards and grown separately on seedlings under the same conditions. For growth of the asexual stages, conidia were harvested from colonies approximately 8 days post inoculation (dpi) by hand shaking infected leaves in distilled water containing 0.005% Tween^{TM} 20. Suspensions of each isolate were made

separately and normalized to a concentration of 200 conidia per 5 µl drop. Germination tests were performed by placing 3 drops of the suspension in a moist chamber for 4 hours and checking percent germination. Only suspensions showing greater than 40% germination were used. For colonies where sexual reproduction was desired, conidial suspensions of both isolates were prepared as above, mixed in equal proportion and vortexed to create one suspension of both mating types.

Colonies for RNA extraction were prepared by placing multiple 5 µl drops of the conidial suspension across the entire surface of the youngest fully expanded leaf of a grape seedling. The suspension was allowed to dry and then the seedlings were placed in a growth chamber at 23° C with a 12 hour photoperiod. When the colonies reached the desired stage, all inoculated leaves were observed under a dissecting scope for uniformity of development. Leaves showing at least 90% uniformity in terms of relative radial growth and appearance of the appropriate sporulation stage (i.e. for colonies in the conidiophore development stage, > 90% of colonies on selected leaves showed conidiophore emergence but no fully developed conidia) were selected for RNA extraction.

RNA Isolation

RNA was extracted from each of four stages: pre-sporulation (3 dpi), at conidiophore initiation (~ 5 dpi), at full sporulation (8 dpi), and at the development of mature ascocarps (approximately 4 weeks post inoculation). All RNA extractions were performed according to Cadle-Davidson *et al* (2009). For the three asexual stages, two independent isolations were performed from each of two isolates. For the sexual stage, four independent isolations were performed. Within each stage, equal amounts of total RNA were pooled from the four independent extractions to form one pool/stage of interest. mRNA was isolated from total RNA using the PolyATtract system (Promega, Madison, WI). The mRNA was not eluted from the beads in the final step, and the beads

were used directly in the iScript™ cDNA Synthesis Kit (Biorad, Hercules, CA) to convert the mRNA into cDNA.

cDNA-AFLP Analysis

The cDNA-AFLP protocol was adapted from Bachem *et al* (1996). cDNA from each stage was digested with *Taq*I followed by an *Ase*I digestion. Resultant fragments were then ligated to adapters for amplification (*Taq*I forward: 5'-GAC GAT GAG TCC GAC-3'; *Taq*I reverse: 5'-CGG TCA GGA CTC AT-3'; *Ase*I forward 5'-CTC GTA GAC TGC GTA CC-3'; *Ase*I reverse 5'-TAC GTA CGC AGT C-3'). Pre-amplification was performed with a *Taq*I+0 primer (5'-GAC GAT GAG TCC TGA CCG A-3') and an *Ase*I+0 primer (5'-CTC GTA GAC TGC GTA CCT AAT-3'). Amplification conditions were as follows: 4 min denaturation at 94° C and then 27 cycles 30 s denaturation at 94° C, 30 s annealing at 55° C, 60 s extension at 72° C. After preamplification, products were diluted to 1 ng/µl (about 5-fold dilution).

Five µl of the diluted preamplifiction product were used as template for the selective amplification. Selective amplification was carried out with 45 primer combinations using two selective nucleotides on each primer (Table 1). The PCR amplification program was as follows: denaturation at 94° C for 4 min, and then denaturation at 94° C for 30 s, annealing at 65° C for 30 s, extension at 72° C for 60 s (13 cycles, scale down of 0.7° C per cycle), followed by 32 cycles of 30 s denaturation at 94° C, 30 s annealing at 56° C, 60 s extension at 72° C.

Selective amplication products were separated by running on a 5% polyacrylamide gel in a Bio-Rad Protean II xi Gel System (Hercules, CA) at 40 W and 45° C for 3.5 hours. Following separation, fragments were visualized by silver staining according to the protocol by Echt *et al* (1996). Fragments of interest were picked from the gel using a sterile pipette tip, transferred to 100 µl distilled water in a microfuge tube, and heated to 95° C for 10 min. One microliter of this eluted DNA was used as a template for reamplification using the pre-amplification primers under the following

Table 1: Listing of all 45 primer combinations used in the cDNA-AFLP analysis of differential expression across four developmental time points in *Erysiphe necator*

TG-GA	CA-GA	GT-AG	GC-AG	CG-GA	GA-AG
TG-GT	CA-GT	GT-TA	GC-TG	CG-GT	GA-TG
TG-GC	CA-GG	GT-CT	GC-CC	CG-GG	GA-GA
TG-GG	CT-GA	GT-CC	GC-GA	GG-AG	GA-GT
CC-AG	CT-GT	GT-GA	GC-GT	GG-TG	GA-GC
CC-GA	CT-GG	GT-GT	GC-GC	GG-GA	GA-GG
CC-GT	GT-GG	GT-GC	GC-GG	GG-GT	GG-GC
GG-GG	GG-AA	GC-AA			

conditions: 4 min of denaturation at 94° C, followed by 8 cycles of 30 s denaturation at 94° C, 30 s annealing at 61° C (reduced by 0.7° C per cycle), 60 s extension at 72° C, followed by 24 cycles of 30 s denaturation at 94° C, 30 s annealing at 56° C, and 60 s extension at 72° C. PCR samples (15 uL) were electrophoresed in a 1% agarose gel, and visualized using UV excitation of SYBR Green in the loading dye. Samples with visible, single bands were purified using Sephadex columns and Sanger sequenced directly using the pre-amplification primers at Cornell University's Core Laboratories Center.

Sequence Analysis

Homology searching by blastx and tblastx was carried out against the National Center for Biotechnology Information (NCBI) database. To increase the probability of finding a match, longer length sequences for the amplified products were found by performing a nucleotide BLAST of each sequence against a conidial stage cDNA library database containing over 32,000 contigs from Roche-454 sequencing of *E. necator* isolate Geneva-14 (G14) (Cadle-Davidson, unpublished data). The cDNA contigs matched in this search were then searched against the NCBI database using blastx and tblastx.

Real Time PCR Confirmation

To confirm differential expression of the identified sequences, quantitative RT-PCR (qRT-PCR) was performed. RNA was isolated from colonies at the 3 dpi, conidiophore initiation, full sporulation, and ascocarp initiation stages. These stages were identical to those used within the cDNA-AFLP experiments, with the exception of the sexually reproducing stage, wherein RNA was isolated from colonies at ascocarp initiation, rather than maturation. The colonies were grown from two fresh isolates of *E. necato*r, F-con2 and G9. For the real time experiments, only one RNA isolation was performed per stage of interest as opposed to the four used in the cDNA-AFLP analysis. Extraction of the RNA, purification of mRNA and synthesis of cDNA was performed as described above. Each pool was then normalized to equal cDNA concentration.

Following purification, the cDNA samples and remaining mRNA were analyzed with *E. necator* primers to confirm the absence of *E. necator* genomic DNA.

After analyzing the sequence data from the cDNA-AFLP experiment, 17 sequencess of interest were identified based on their expression pattern and match in the BLAST searches. Primers for real time were designed for all 17 sequences and tested for quantitative response to gDNA and specificity by melt curve analysis. Three technical replications were performed on all melting curves. Following this initial analysis, six sequences were selected for further analysis based on their melting curves.

Three replications of cDNA from each time point were run for each gene fo interest and original concentration were estimated based on the standard curve.

Results

A total of 45 primer combinations were used for cDNA-AFLP analysis, giving 21-64 bands, or transcript derived fragments (TDFs), ~100-800 bp in size, per primer combination (Figure 4). Across all 45 primer combinations a total of approximately 1,600 TDFs were visualized. Within these, 620 TDFs (39%) were polymorphic across one or more of the four stages, suggesting differential expression. Of these polymorphic bands, 242 were directly sequenced. The remaining bands out of the 620 TDFs either gave poor product upon reamplification or gave a degraded product when sequenced. When searched against the NCBI database using blastx and tblastx, 45 (20%) of these had a significant match (E value $\leq$ 1 E-03). Only one sequence matched to *Vitis vinifera*, indicating the extraction method was successful in targeting *Erysiphe* specific RNA. To improve the ability to find matches, the short length TDFs were searched against an *E. necator* cDNA library. Of the 242 sequences searched against the library 162 (67%) showed significant matches to 1-9 cDNA contigs. There were 121 contigs that showed a significant match when searched against the NCBI database, again using blastx and tblastx. Where the search of the original TDF had matched multiple contigs in the cDNA

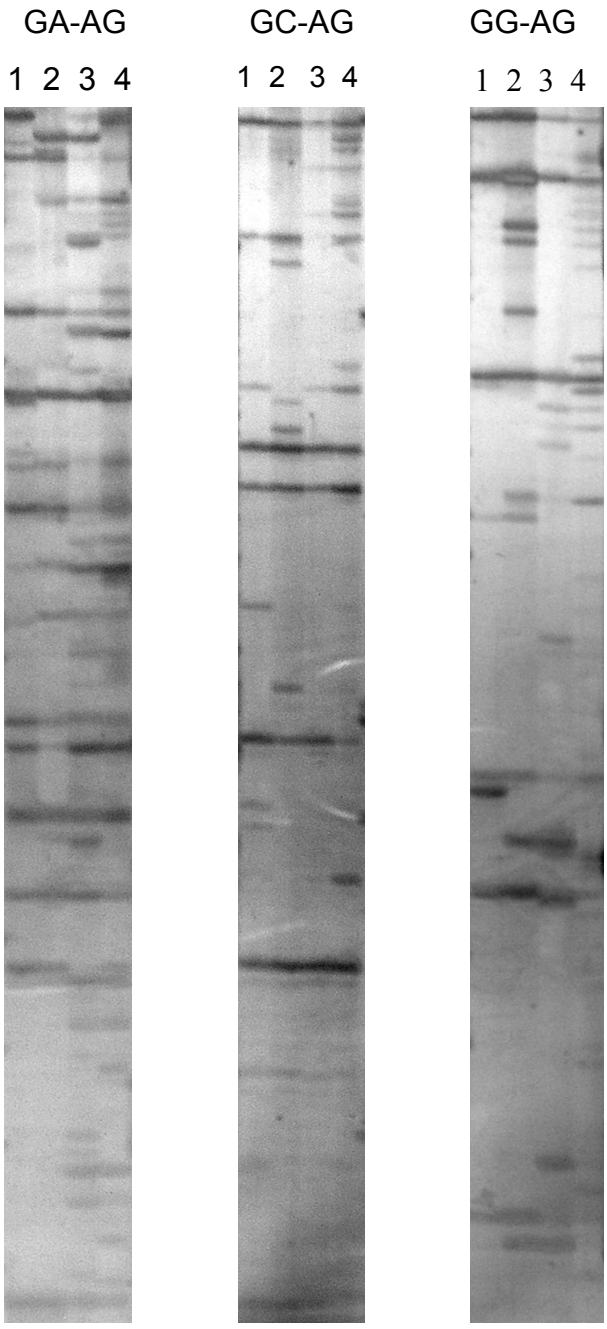


Figure 4: Representation of differential expression of *Erysiphe necator* transcripts across four developmental stages for three cDNA-AFLP primer combinations. Transcript profiles across three primer combinations are shown. Primer combinations are given by selective nucleotides in the following 5'-3' format: *Ase*I primer-*Taq*I primer The cDNA pools were as follows: 1) pre-sporulation, 2) conidiophore initiation, 3) full sporulation, and 4) ascocarp maturation.

library, the group of matching contigs matched similar sequences in the NCBI database, indicating that these multiple hits were members of a gene family.

Functional classification of sequences

Based upon searches of the relevant literature and analysis of the sequences, five classes of differentially expressed fragments were identified. The most interesting of the matches to the NCBI database based on the match and the expression pattern are listed in Table 2 with the corresponding sub-cellular location of sequencess of particular interest depicted in Table 3 (a complete list of sequences is available in Appendix I). Of the *E. necator* sequences assigned to each class, 45% of the sequences had no significant match in the databases searched (Figure 5). An additional 16% matched hypothetical fungal protein sequences of unknown function. Among the sequences that matched sequences of predicted or known function, the largest class, representing 13% of differential transcripts, were sequences involved in metabolism. Other groups of interest, each representing 2-8% of the TDFs, included transcription factors, signaling proteins, and transport proteins.

Confirmation of differential expression by real-time RT-PCR

Normalized expression confirmed that the cDNA pools were free of contaminating genomic DNA (Figure 6). The results from five of the six sequences showed good agreement with what was expected from the cDNA-AFLP analysis (Figure 7). Where expression was expected to be highest at full sporulation, expression at ascocarp initiation was also high. In general, expression was lowest at pre-sporulation and conidiophore initiation. The single gene with expected expression in the early stages, 3182, showed low expression at pre-sporulation and conidiophore initiation and high expression in later stages.

Discussion

The cDNA-AFLP method of analyzing transcriptional regulation across developmental stages has been described as useful for comparisons across multiple

Table 2: Functional classification of selected differential transcripts. Primer combinations are given by selective nucleotides in the following format: *Ase*I primer-*Taq*I primer. Each TDF was given a number based on the primers used and the number band within each primer combination. TDFs with no match to the cDNA library are scored with a * in the blast search against the library. The E-values of the match are listed as the direct search of the TDF sequence against the NCBI database. The stages of expression are represented as follows: 1) pre-sporulation, 2) conidiophore initiation, 3) full sporulation, and 4) development of mature ascocarps.

Primer Comb.	TDF No.	Expressed In:	Blast score in search against cDNA contigs	Annotation	Blast Score
				Metabolism	
CA-GA	9-13-28	3	6 E-27	Lysine synthesis/Lys2 (XP_002143711)	9 E-132
CC-GA	11-13-11	1	3 E-92	Spermine/spermidine synthase (EEH21650)	1 E-136
CC-GA	11-13-20	4	4 E-44	Acetyl coA synthase (XP_001935668)	1 E-52
GA-GG	13-16-17	1	5 E-37	Acetolactase synthase (XP_962652)	0
GA-GG	13-16-18	1-3	5 E-34	Pap2 domain protein (XP_001931173)	1 E-72
TG-GT	8-14-12	1-3	7 E-29	Cytochrome P450 (XP_755288)	1 E-60
GT-GG	14-16-19	1-3	4 E-34	Vacuolar ATP synthase subunit B (XP_001557622)	0
GC-AG	15-4-5	1 & 4	1 E-47	D-3 phosphoglycerate dehydrogenase (XP_0022382929)	4 E-162
GC-GT	15-14-33	1-2	3 E-25	Chitin Synthase (XP_956331)	0
GG-AG	16-4-7	2-4	1 E-66	Protoheme IX farnesyl transferase (EER45220)	3 E-72
GG-GC	16-15-11	3-4	1 E-47	Alcohol dehydrogenase (EEH49979)	8 E-47
TG-GT	8-14-11	1-3	6 E-54	Actin polymerization protein/Bzz1 (XP_749256)	5 E-87
GG-GG	16-16-21	1-3	3 E_23	Fatty aldehyde dehydrogenase (XP_001932922)	3 E-165
GG-AG	16-4-3	1 & 4	4 E-40	Caleosin domain protein (XP_001932418)	2 E-53
TG-GA	8-13-22	1	2 E-39	Elongation Factor 3 (XP_001593480)	0
GT-GT	14-14-15	4	2 E-56	Gamma tubulin (XP_001903940)	6 E-76
				Transcription Factors/DNA Binding	
CG-GA	12-13-18	1	1 E-43	C2H2 zinc finger domain (XP_002482718)	6 E-32
GT-GA	14-13-9	1-2	4 E-85	C2H2 zinc finger domain (XP_002382564)	0
GT-AG	14-4-18	4	5 E-27	C2H2 transcription factor RfeC (XP_002481027)	4 E-25
GT-GT	14-14-11	2-4	7 E-65	Gal4-like transcriptional activator (AAG25917)	4 E-117
GC-GT	15-14-31	2	5 E-6	Transcriptional activator Spt7 (EEHO4734)	3 E-51

Table Two (Continued)

GG-GG	16-16-7	2-4	3 E-38	Transcriptional elongation factor Spt6 (EEH34974)	0
GG-AG	16-4-2	1 & 4	3 E-56	FlbD (EEd33126)	1 E-4
GG-TG	16-8-9	2-3	4 E-32	Ring finger domain protein (XP_00125767)	7 E-130
GC-GA	15-13-10	1-3	2 E-41	CobW domain protein (XP_002384953)	3 E-170
GT-TT	14-6-8	2-4	5 E-47	Mads box transcription factor (XP_001935687)	7 E-46
GC-GG	15-16-10	1-3	6 E-51	Histone transcriptional regulator (CAE85596)	2 E-34
CA-GA	9-13-17	4	3 E-9	PHD and ring finger domain protein (EER42091)	1 E-43
				Signaling	
TG-GA	8-13-19	1-2	4 E-50	Calcium permease (XP_001547054)	1 E-73
GA-GA	13-13-3	2	1 E-179	Integral membrane protein/Pth11 homologue (AAD30437)	6 E-64
CA-GA	9-13-24	4	2 E-24	Catalase peroxidase (Q8X1N3)	0
CA-GT	9-14-35	3	3 E-38	Cystolic regulator Pianissimo (XP_001938513)	0
GA-GT	13-14-14	4	1 E-59	Caseine kinase I (XP_001272602)	0
CG-GA	12-13-13	1-3	9 E-39	Serine/threonine protein kinase (XP_001548642)	1 E-171
CA-GA	9-13-27	2	2 E-24	Kelch repeat protein (EEE22934)	3 E-34
				Transport/Cell Membrane Proteins	
TG-GG	8-16-11	2-3	4 E-63	RAN homologue (XM_001594666)	6 E-114
GA-GA	13-13-12	2-3	3 E-35	Coatomer subunit alpha (AAC18088)	2 E-116
GT-GT	14-14-13	4	2 E-38	SAC3-GANP domain protein (EEH38661)	3 E-77
GC-GA	15-14-14	1-2	1 E-17	Importin beta-3 subunit (XP_001264986)	0
TG-GT	8-14-22	2-4	3 E-51	Stomatin family protein (XM_001594877)	2E-149
GT-GT	14-14-12	3-4	1 E-40	Integral membrane protein (XP_0022376767)	8 E-66
GT-TG	14-8-8	1 & 4	3 E-64	PRELI/MSF1 domain protein (EER25643)	2 E-57
GT-TA	14-5-1	1	*	MFS monocarboxylic acid transporters (EDP52060)	5 E-07
				Protein Maintenance and Degradation	
TG-GA	8-13-21	3	7 E-46	ER-associated proteolytic system protein DER1 (XP_747611)	3 E-77
TG-GT	8-14-21	2	9 E-39	Heat shock protein 70 (EU311400)	0
GA-GG	13-16-9	3	2 E_58	F Box domain protein (XP_001272021)	2 E-105

Table 3: The expression pattern of select sequences is shown for each stage in its corresponding sub-cellular location. Sequences expressed at each stage are shown in bold, Sequences were selected for addition to this table based on their expression pattern and predicted ties to developmental control.

	Pre-sporulation	Conidiophore initiation	Full Sporulation	Ascocarp Maturation
Nucleus	**C2H2 Transcription Factors**	**C2H2 Transcription Factors**	C2H2 Transcription Factors	C2H2 Transcription Factors
	Ring Finger Domain	**Ring Finger Domain**	**Ring Finger Domain**	**Ring Finger Domain**
	CobW Domain	CobW Domain	CobW Domain	CobW Domain
	FlbD	FlbD	FlbD	**FlbD**
	RAN	**RAN**	**RAN**	RAN
	Importin beta subunit	**Importin beta subunit**	Importin beta subunit	Importin beta subunit
	Sac3/GanP	Sac3/GanP	Sac3/GanP	**Sac3/GanP**
Cytoplasm	F-Box Domain	F-Box Domain	**F-Box Domain**	F-Box Domain
	Kelch Repeat Protein	**Kelch Repeat Protein**	Kelch Repeat Protein	Kelch Repeat Protein
	Pianissimo	Pianissimo	**Pianissimo**	Pianissimo
Lipid Body	**Caleosin**	Caleosin	Caleosin	**Caleosin**
	Cytochrome P450	Cytochrome P450	**Cytochrome P450**	Cytochrome P450
Cell Membrane	**Pap2 Domain**	**Pap2 Domain**	**Pap2 Domain**	Pap2 Domain
	Stomatin Family	**Stomatin Family**	**Stomatin Family**	Stomatin Family
	Putative GPCR	**Putative GPCR**	Putative GPCR	Putative GPCR
Golgi Body	**Caleosin**	Caleosin	Caleosin	**Caleosin**
Endoplasmic Reticulum	Proteolytic System Protein	Proteolytic System Protein	**Proteolytic System Protein**	Proteolytic System Protein

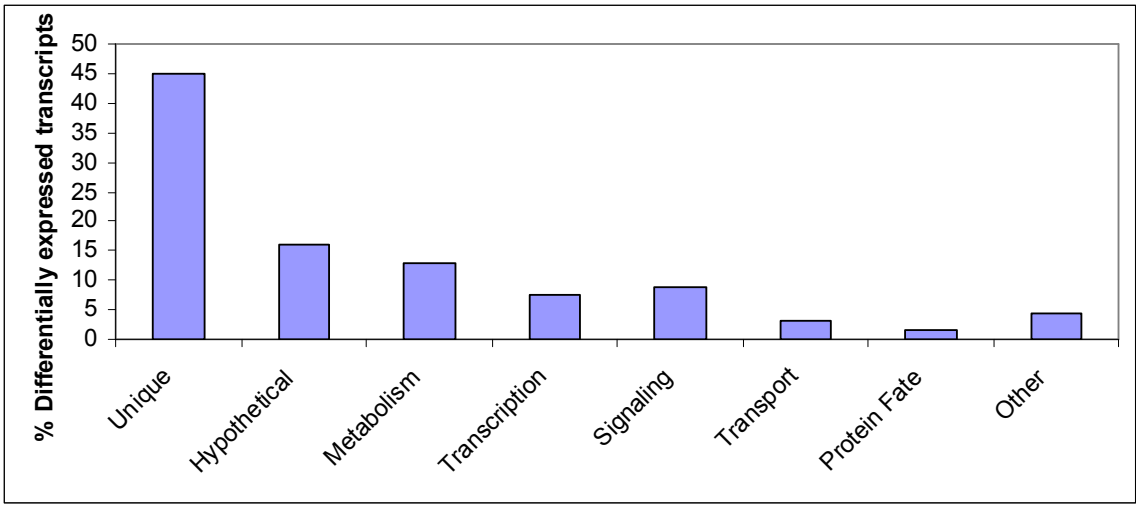
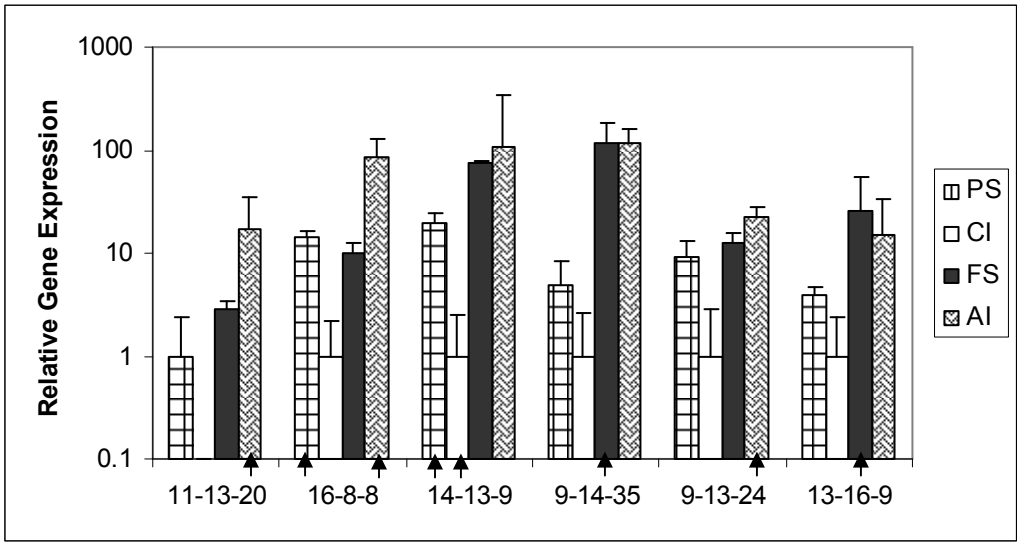


Figure 5: *Erysiphe* transcripts showing differential expression across four sporulation time points. Frequency of differentially expressed sequences within each category expressed as percentage within total number of differential sequences.

Figure 6: Confirmation **of *Erysiphe necator* RNA extraction**. The marker (M) is a 1 kb ladder (Promega, Madison, WI). Lanes 1, 3 and 5 represent RT-PCR using *E. necator* primers on RNA extractions from pre-sporulating, conidiophore initiation, and full sporulation colonies respectively. Lanes 2, 4, and 6 represent PCR without reverse transcription using the same primers on the same RNA extractions. Lane 7 represents RT-PCR using a separate set of *E. necator* primers on RNA extracted from ascocarp initiation colonies. This primer was used on this pool as the original primers gave no product from this stage. Lane 8 represents PCR without reverse transcription on the ascocarp initiation RNA. Lanes 9 and 10 represent the no template controls (NTC) for the grape-c1339 and lrc148 primers respectively.



TDF Code	Expected Expression	Annotation
11-13-20	Ascocarp Initiation	Acetyl CoA FacA Synthase
16-8-8	Pre-sporulation, Ascocarp Initiation	MSF1 Domain Protein
14-13-9	Pre-sporulation, Conidiophore Initiation	C2H2 Zinc Transcription Factor
9-14-35	Full Sporulation	Pianissimo
9-13-24	Ascocarp Initiation	Catalase peroxidase
13-16-9	Full Sporulation	F-Box Domain Protein

Figure 7: qRT-PCR Analysis. qRT-PCR analysis for 6 selected genes across four time points in *E. necator* sporulation. Expected expression was based on the cDNA-AFLP analysis. The stages are as follows from left to right: pre-sporulation (PS), conidiophore initiaton (CI), full sporulation (FS) and ascocarp initiation (AI). All data were normalized to one expression unit. Data represent fold change of gene expression across all four time points. The stage with the highest expression is marked with an ↑. Bars represent the standard error calculated on 3 technical replicates.

treatments in non-model species (Polesani *et al*, 2008) and particularly appropriate in studies of obligate biotrophs where little sequence info is available (Van der Biezen *et al*, 2000). Our results using this technique in *E. necator* demonstrated that it was a successful tool in morphogenesis studies in this pathosystem. Using 45 primer combinations, we were able to visualize approximately 620 differentially expressed fragments, out of 1,600 total fragments. While this level of differential expression is high for this type of study, it is not a surprising result considering the large physical changes of the fungus from purely vegetative growth to asexual and ultimately sexual reproduction that were studied. In addition, developmental events related to host infection occur at the same time.

Of the sequenced fragments, 45% showed no significant homology to known sequences. This result additionally underscores the appropriateness of using a platform where prior sequence knowledge was not required. Analysis of those sequences which showed similarity to previously described sequences revealed several patterns in gene expression, described by category here.

Metabolism

The largest class of identified genes was involved in cell metabolism. As seen in Table 2, a number of the metabolism genes were expressed early on in the vegetative and asexual stages of growth, but were switched off in late stages of infection. In *E. necator*, ascocarps are produced later in the growing season as a means of surviving cold winter climates (Gadoury and Pearson, 1988). It is not surprising then to find that many basic synthesis and metabolism genes would be down-regulated as the fungus prepares for the end of the growing cycle.

Several of the identified genes have interesting implications for regulation of sporulation. Three TDFs matched structural genes: chitin synthase (15-14-33), actin polymerization (8-14-11), and gamma tubulin (14-14-15). Structural proteins, in particular chitin synthase, have been shown to be differentially regulated across different

cell types, and fine control of cell wall synthesis is predicted to play an important role in functional spore production (Fischer and Kües, 2006). Several other genes have links to secondary metabolism, which has a documented connection with asexual development (Yu and Keller, 2005). Aldehyde dehydrogenase, for example, is linked with the production of secondary metabolites like pigments in *Neurospora* (Sandmann *et al*, 2008), which is linked with asexual development through dual coordination of pigment production and conidiation (Yang and Borkovich, 1999).

Most interesting within this group however, were the genes linked with lipid metabolism. Although fungi use a variety of compounds as sporulation-specific signaling molecules, lipid signals, particularly the oxylipins, have demonstrated roles in coordinating fungal development. Recent investigations in our lab showed that initiation of sporulation may be coordinated by a quorum sensing mechanism (Chapter One). Although quorum sensing phenomena have not been well characterized in fungi, oxylipin farnesol has been shown to act as a quorum sensing molecule in dimorphic fungi (Nickerson *et al*, 2008). A second group of oxylipins, the psi (precocious sexual inhibitor) factors, coordinate the switch between vegetative, asexual and sexual development in *A. nidulans* (Tsitsigiannis *et al*, 2004).

Three of the differential sequences showed links to lipid metabolism. The first showed a relatively strong hit to a Pap2 domain protein (13-16-18). The phosphatidate phosphatase (PAP) enzymes are involved in the generation and degradation of lipid-signaling molecules (Carman and Han, 2006). In yeast, the Pap2 domain proteins are additionally linked to nutrient stress responses (Han *et al*, 2001). Sporulation in response to nutrient stress is a common response in filamentous fungi, and it may be the Pap2 domain proteins are responsible for the coordination of some type of lipid signal that stimulates this response.

In *Aspergillus*, the oxylipin psi-factors are produced by the Ppo enzymes. Recent work with PpoA revealed that this protein is a fusion of a fatty acid heme dioxygenase

and a Cytochrome P450 domain (Brodhun *et al*, 2009). Our investigations also revealed a match to a Cytochrome P450 (8-14-12) expressed during vegetative and asexual growth stages. It may be that this sequence is part of a larger protein responsible for the production of a similar signal. Cytochrome P450 has also been shown to be required for the production of certain secondary metabolites, such as aflatoxin in *Aspergillus*, and it may be that this protein provides a similar function in *E. necator*.

The final hit with links to lipid metabolism was the caleosin domain protein (16-4-3). Caleosins are heavily associated with the maintenance of storage lipid bodies in plant seeds and are upregulated during seed development (Chen *et al*, 1999). The caleosin sequence in *E. necator* was upregulated both early and late in development. It could be that caleosins are produced early in infection to maintain lipid bodies upon which germinating spores feed until successful infection occurs and then again during sexual reproduction to produce storage bodies for the developing ascospores. If so, it is conceivable that these lipid bodies contain lipid signals for the repression of asexual sporulation. While the fungus relies upon the lipid stores for energy early on in infection, these lipid signals could keep growth purely vegetative until the lipid stores are exhausted and replaced by constant nutrition from the host plant. As conidiation is an energy-intensive process, a developmental program which halted conidiation until host-resources could be relied upon would be a distinct advantage. Proteins responsible for repression of asexual development like PpoA are known to localize to lipid bodies (Tsitsigiannis *et al*, 2004). Such lipid bodies may need caleosin for stabilization. Caleosins are also predicted to have functions in lipid body and vesicle trafficking and therefore may also play a role in the export of some anti-sporulation signal (Hernandez-Pinzon *et al*, 2001).

Signaling

It was within the genes showing roles in signaling where the greatest similarity between *E. necator* sequences and the model systems is seen. In the model fungi, heterotrimeric G-protein signaling has a well established role in coordinating multiple

responses, including sporulation (for review, see Yu *et al*, 2006). We have uncovered several elements of G-protein signaling that indicate that the same basic elements may provide the basis for developmental control in *E. necator.*

TDF 13-13-3 showed moderate similarity to homologues of Pth11. Pth11 has been predicted to encode a G-protein coupled receptor (GPCR) in *Magnaporthe grisea* and may play a role in pathogenesis related morphogenesis (Odenbach *et al*, 2007). The corresponding gene in *E. necator* was expressed only at conidiophore initiation and may have some function as a receptor of sporulation-specific signals. If so, the fact that it was expressed at such a transient stage may explain some results achieved in earlier studies dealing with initiation of sporulation. In specific, Mooney and Yager (1990), in their experiments into light-induced conidiation in *A. nidulans*, demonstrated that light must be provided within a very narrow six-hour window or asexual sporulation will not occur. If the receptor molecules for sporulation signals such as light are only transiently expressed, it may explain why such a tight window exists for the supply of light in other fungi.

Several other identified sequences showed some role in G-protein signaling. The first of these is *Pianissimo* (9-14-35). In *Dictyostelium*, Pianissimo (PiaA) is required for the G-protein linked cAMP signaling. Disruption of homologues of PiaA in *S. cerevisiae* and *S. pombe* result in lethality, suggesting the protein has some conserved role in other fungi (Chen *et al*, 1997).

Although kelch-repeat proteins have numerous functions in eukaryotic cells, it may be that the kelch repeat sequence found in this study has some role in G-protein signaling as well. In *Aspergillus*, the primary G α subunit that coordinates asexual development is FadA. FadA transmits its signal to the cell through the cAMP/Protein Kinase A pathway. (Shimizu and Keller, 2001). Peeters *et al* (2007) demonstrated that two kelch repeat proteins, Krh1p and Krh2p, can bypass the need for cAMP and directly link G α subunits with protein kinase A. The kelch-repeat sequence in *E. necator* was expressed at conidiophore initiation alone, coincident with the putative GPCR sequence,

thus suggesting that it indeed may have some role in transmitting signals received through this GPCR.

Transcriptional Regulators

After genes involved in metabolism and signaling, our largest discovered group was those involved transcriptional regulation and DNA binding. While sequences in this group showed upregulation across each of the four time points tested, upregulation appeared most often at the conidiophore initiation stage indicating large changes in gene regulation at this time point.

Within this group, we did see one weak match to a known regulator of sporulation, FlbD. In *Aspergillus*, *flbD* encodes a myb-like DNA binding protein that is necessary for early-stage conidiation events. Unlike 16-4-2, the possible match in *E. necator*, which showed upregulation in the vegetative and sexual stages, *flbD* exhibits constant expression throughout the *A. nidulans* life-cycle and is thought to be controlled through post-transcriptional modification (Wieser and Adams, 1995). If indeed this sequence is a homologue of *flbD*, it appears it may possess a different function in *E. necator*.

A number of sequences also showed similarities to Cys_2-His_2 (C2H2) transcription factors (12-13-18, 14-13-9. and 14-4-18). One of the best characterized of the *Aspergilllus* sporulation-specific transcription factors, *brlA*, also falls within this class (Adams *et al*, 1998). More recently, a RING-finger domain protein, crgA, was shown to play a role in blue light stimulated asexual sporulation in *Mucor* (Nicolas *et al*, 2008). Sporulation in *E. necator* is also stimulated by light (Chapter 1). The appearance of both C2H2 and RING-finger domain proteins in *E. necator* may indicate that similar proteins may be acting in powdery mildew.

Cell membrane/transport

Transport proteins have been studied in other fungi and have implications in asexual development. The velvet (VeA) complex in *Aspergillus* plays a critical role in

integrating multiple signals in controlling asexual v. sexual development and in production of secondary metabolites. The velvet protein represses asexual development in the dark and moves in and out of the nucleus in response to light (Bayram *et al*, 2008). Araújo-Bazán *et al* (2009) recently demonstrated that importin α interacts with VeA and importin α mutants show impaired nuclear transport of VeA. We have found a sequence showing similarity to the importin β subunit expressed in early stages. The two subunits act as a heterodimer, with both necessary for proper function. The importin-like sequence may have some function in importing nuclear signals similar to VeA in *E. necator*.

Related to the function of importin is Ran, a small GTP-ase. The importin heterodimer releases its contents in the nucleus upon disassociation of the heterodimer. This is stimulated by the interaction of the β subunit with Ran (Araújo-Bazán *et al*, 2009). The sequence showing some similarity to Ran (8-16-11) was expressed at conidiophore initation and full sporulation as opposed to the expression pattern of the importin-like signal which showed upregulation at pre-sporulation and conidiophore initiation. Studies in *Exserohilum* also showed upregulation of Ran during conidiation and not in earlier stages (Flaherty and Dunkle, 2004). It may be that nuclear transport is impaired until initiation of sporulation and the Ran-importin interaction is important for import of complexes which trigger, rather than repress sporulation.

In addition to proteins involved in nuclear transport, we found a number with membrane-associated functions. We found one sequence with similarity to stomatin family proteins (8-14-22). The stomatin family proteins have roles in stabilization of proteins in membranes. It may be that this sequence has some function in stabilizing other receptors or transport proteins in the membrane.

Protein Maintenance/Degradation

The final class identified was those involved in protein fate. Studies in other fungi have suggested that protein turnover may have some role in control of asexual

sporulation. Recent studies in *Neurospora* have indicated that genes involved in protein degradation are upregulated during asexual sporulation (Kasuga and Glass, 2008). The Cop9 signalosome, which has functions in targeted protein degradation, coordinates development in *Aspergillus* (Busch *et al*, 2003) and plays a role in regulation of PpoA expression (Tsitsigiannis *et al*, 2004). F-box proteins, which act as substrate receptors for ubiquitin ligase, have also been proven necessary for proper cell division and sexual spore development in *Aspergillus* (Krappmann *et al*, 2006).

Similar to the work done in *Neurospora*, we saw upregulation of protein degradation genes at asexual sporulation. Two sequences, a proteolytic system protein (8-13-21) and an F-box domain protein (13-16-9) showed upregulation at full conidiation alone. It could be that the upregulation of proteolytic systems is necessary for turnover of protein complexes responsible for the suppression of asexual development. Although no turnover points have yet been discovered, the developmental regulator and suppressor of asexual development VeA contains PEST domains for rapid turnover indicating degradation of VeA is important for development control (Calvo, 2008). Targeted degradation of complexes like VeA may be assisted by upregulation of the protein turnover genes.

Real Time Confirmation

Confirmation by qRT-PCR analysis proved generally successful, with 5 out of 6 genes showing agreement with expected results. However, some artifacts in the results suggest better optimization of the procedure is required. In places where expression was expected to be highest at full sporulation, high levels of expression were also seen at ascocarp initiation. This is likely the result of RNA being isolated from different stages in the qRT-PCR work versus the original cDNA-AFLP analysis. As RNA was extracted from ascocarp initiation, as opposed to fully mature ascocarps in the cDNA-AFLP work, sporulation had not yet completely shut off and some overlap between expression at full sporulation and this stage was to be expected.

More troubling is the observation that expression was nearly always the highest at full sporulation and ascocarp initiation, even in genes like 3182 where expression was expected to be higher earlier in development. This indicates that a better normalization procedure than use of equal amounts of total cDNA could improve the expression analysis.

Conclusions

The work in this paper represents the first investigation into differential gene expression during morphogenesis in *E. necator*. Our results have shown some similarity between control of development in *E. necator* and the models from *Aspergillus* and *Neurospora*, particularly in the areas of G-protein signaling, transcriptional regulation and nuclear transport.

The largest group of sequences, however, was those with no match to known sequences. Forty-five percent of sequenced TDFs showed no significant match. This indicates that while control of sporulation in *E. necator* may share some basic elements with established systems, it is likely that there are significant points of divergence as well. Given the differences in growth and reproduction between the powdery mildews and the model fungi, this is not surprising. Most significantly, the powdery mildews are obligate biotrophs. It seems likely that they have a more intimate connection with their host species and may rely on the host for developmental cues in ways fungi like *Aspergillus* do not. In addition, *E. necator* is heterothallic unlike the self-fertile *A. nidulans*. Sexual reproduction is initiated upon contact between hyphae of opposite mating type, not in response to environmental triggers such as lack of light. It is possible that control of asexual versus sexual development is handled by differing complexes.

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CONCLUSIONS

Light and colony density may act as triggers of asexual development in *Erysiphe necator*

Chapter one describes a series of experiments establishing evidence for a signaling process that triggers conidiation in *E. necator*. The basic model follows that established in other systems. Colonies exhibit purely vegetative growth for a period of approximately five days whereupon conidiophores emerge and asexual sporulation commences. Experiments with light signaling have established that light is required for conidiation, but light provided early in development (before 3 dpi) has no ability to trigger asexual sporulation. This follows what was demonstrated in *A. nidulans*, where light could only promote conidiation when supplied after colonies became competent for sporulation (Mooney andYager, 1990).

More intriguing are possible links between colony density and asexual development. A clear relationship was demonstrated between inoculum density and latent period, suggesting that conidiation is potentially triggered by a quorum-signaling like mechanism. If colony density does act as a trigger for asexual development, it could have a profound impact on current understanding of powdery mildew epidemics, particularly in under-estimation of latent periods.

Recent observations have suggested that density may play a role in the suppression of conidiation as well as in its initiation. Experiments in mating compatible isolates revealed that when very low densities of inoculum are used (~5 germinable conidia per 5 ul drop) asexual sporulation is entirely suppressed: colonies develop hyphae and eventually ascocarp initials, but never display upright conidiophores. This is in contrast to what is observed in higher inoculum density where conidiophores initially develop, produce a crop of conidia, and then eventually wither as the ascocarps mature. It may be that in denser colonies, the conidiation-initiation signal is strong enough to at least initially overcome the suppressing signal produced by mating. As ascocarps

develop, however, the suppressing signal may grow in strength to the point where it overcomes the conidiation signal and resulting in total suppression of asexual reproduction.

Genes associated with developmental processes in *E. necator* show some similarity to genetic models from established systems

Chapter two outlines the investigations into sequences associated with several time points in development in powdery mildew. The experiments demonstrated that cDNA-AFLP is an appropriate technique for identifying such sequences in pathogens like *E. necator* where little sequencing information is available. Using cDNA-AFLP, we were able to identify 231 differentially expressed sequences. Forty-five percent of these showed no homology to known sequences, indicating that *E. necator* may be using a number of previously uncharacterized genes in coordinating development.

Some basic points of similarity between discoveries in *E. necator* and other systems were seen however. Some elements of G-protein signaling were revealed as well as important proteins in nuclear transport, both of which have established roles in asexual development in *A. nidulans* (Yu, 2006; Aráujo-Bazán *et al*, 2009). Significant overlap was also seen in the types of transcriptional activators identified in *E. necator* and other fungi. The commonalities in developmental control between powdery mildew and other systems suggests that some information learned in model fungi may be applicable to powdery milew, despite the fact that *E. necator* is not a close relative to the models *A. nidulans* and *N. crassa*.

Suggestions for future investigations in development in *E. necator*

Chapter one revealed a number of clues about when a potential signal may be acting to promote asexual sporulation in powdery mildew. The microsurgery experiments suggested that the signal is sent somewhere between 4 and 5 dpi. The light experiments further revealed that light provided up until 3 dpi has no later affect on sporulation, indicating that light is necessary at some later developmental point. Further

light experiments could be used to more precisely identify when colonies become competent for sporulation in a manner similar to that used by Mooney and Yager (1990).

Currently, efforts are underway to sequence the *E. necator* genome. These efforts could greatly simplify further efforts in identifying genes controling development. As discussed in chapter two, having longer length sequences available from an *E. necator* cDNA library greatly aided annotation of identified TDFs. However, 33% of the sequences had no significant match in the cDNA library. Longer matches to these remaining TDF sequences in a future whole-genome sequence for *E. necator* may aid in annotation of these sequences, most of which currently have no significant match in the NCBI databases.

In silico investigations using genome sequence information have also proved useful in other model systems. Genome sequence information has been used to uncover elements of G-protein signaling such as GPCRs, subunits of heterotrimeric G proteins and homologues of important proteins like VeA in other fungi (Lafon *et al*, 2006; Bayram *et al*, 2007). It seems likely that similar investigations will have some success in *E. necator* as well.

At the present time, although there are some intriguing hints as to how sequences associated with developmental processes may be acting in *E. necator*, functional analysis is still required. This is complicated in *E. necator* due to the obligate nature of its growth. Genes important for sporulation cannot be constitutively silenced or we will lack the ability to propagate the mutants. Silencing under an inducible promoter of some sort will be required. Silencing sporulation-specific genes under inducible promoters has been an important tool in characterizing conidiation genes in *Aspergillus* (Barton and Prade, 2008) and could play a critical role in functional characterization in *E. necator*.

Induced silencing may provide an avenue for identifying chemical compounds which trigger or suppress sporulation as well. As a part of the investigations that went into this thesis, efforts were made to extract compounds with some observable effect on

sporulation. Extracts with water proved to have no effect whereas extracts with methanol, ethanol and ethyl acetate were largely lethal to the colonies. It may be that creating a sporulation mutant and then looking for what compounds are lacking as compared to the wild type will be a more effective means of identifying critical compounds in *E. necator* (Donna Gibson, personal communication).

Functional analysis of genes as well as potential isolation of chemical triggers or suppressors of sporulation will potentially open up new means of disease control for grapevine powdery mildew. If the suppressors are amenable to large scale manufacture, they may be directly applied in a spray. It could also be that transgenic grapes with the potential to interfere with signaling processes may be created. Given the nature of the wine industry and the resistance to genetically modified crops in critical portions of the market, it may also be that the identification or the creation of a biocontrol with the ability to interfere with signaling may be a better avenue for disease control. Potentially, a combination of the above techniques could be used, providing a multi-level strategy for repressing sporulation and therefore interfering with disease spread. Such a strategy may prove the most effective means for controlling a pathogen like powdery mildew which shows such adaptability in overcoming disease control efforts and which can cause such significant damage at low levels of infection.

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APPENDIX ONE: LIST OF ALL DIFFERENTIALLY EXPRESSED SEQUENCES AND THEIR ANNOTATION

Primers[a]	Band Id.[b]	TDF No.	Stage[c] 1 2 3 4	E-value versus cDNA library[d]	Annotation[e]	E-value NCBI[f]
TG-GA	8-13-4	1	x	*	No significant match	
TG-GA	8-13-10	2	x	7 E-34	Hypothetical fungal proteins	4 E-79
TG-GA	8-13-13	3	x	8 E-52	No significant match	
TG-GA	8-13-16	4	x	8 E-52	No significant match	
TG-GA	8-13-17	5	x x	1 E-44	No significant match	
TG-GA	8-13-19	6	X x	4 E-50	Calcium permease	1 E-73
TG-GA	8-13-20	7	x	4 E-51	No significant match	
TG-GA	8-13-21	8	x	7 E-46	ER associated proteolytic protein DER1	3 E-77
TG-GA	8-13-22	9	x	2 E-39	Elongation factor 3	0
TG-GT	8-14-4	10	x x X	0.097	No significant match	
TG-GT	8-14-11	11	x x x	6 E-54	Actin polymerization protein	5 E-87
TG-GT	8-14-12	12	x x x	7 E-29	Cytochrome P450	1 E-60
TG-GT	8-14-19	13	X x	3 E-57	Hypothetical fungal proteins	4 E-175
TG-GT	8-14-21	14	x	9 E-39	Heat shock protein 70	0
TG-GT	8-14-22	15	X X x	3 E-51	Stomatin family proteins	2 E-149
TG-GT	8-14-25	16	x x	*	No significant match	
TG-GT	8-14-26	17	x	3 E-38	Hypothetical fungal proteins	4 E-79
TG-GG	8-16-1	18	x x	2 E-68	RAN homologue	6 E-114
TG-GG	8-16-5	19	x x	4 E-27	RAN homologue	6 E-114
TG-GG	8-16-7	20	x x x	4 E-66	Glycine dehydrogenase	2 E-125
TG-GG	8-16-8	21	x x	4 E-48	No significant match	
TG-GG	8-16-9	22	x	*	No significant match	
TG-GG	8-16-11	23	x x	2 E-68	RAN homologue	6 E-114
TG-GG	8-16-12	24	x	4 E-10	No significant match	
TG-GG	8-16-13	25	x	4 E-39	Hypothetical fungal proteins	3 E-12
TG-GG	8-16-14	26	x X x	*	Splicing factor 3	2 E-12
TG-GG	8-16-15	27	x	4 E-26	Hypothetical fungal proteins	4 E-30
CA-GA	9-13-5	28	X x x X	2 E-29	Hypothetical fungal proteins	1 E-108

Primers[a]	Band Id.[b]	TDF No.	Stage[c] 1	2	3	4	E-value versus cDNA library[d]	Annotation[e]	E-value NCBI[f]
CA-GA	9-13-16	29	x			x	9 E-78	Hypothetical fungal proteins	1 E-108
CA-GA	9-13-17	30				x	3 E-9	PHD/Ring Finger domain	1 E-43
CA-GA	9-13-24	31				x	2 E-24	Catalase peroxidase	0
CA-GA	9-13-27	32		x			2 E-24	Kelch repeat proteins	3 E-34
CA-GA	9-13-28	33			x		6 E-27	Lys2 analogue	9 E-132
CA-GT	9-14-26	34			x		9 E-29	No significant match	
CA-GT	9-14-28	35	x				8 E-11	Retrotransposable element	6 E-17
CA-GT	9-14-30	36	x	X			*	No significant match	
CA-GT	9-14-32	37		x			5 E-31	Retrotransposable element	6 E-17
CA-GT	9-14-33	38	X	X		x	1 E-7	Hypothetical fungal protein	1 E-38
CA-GT	9-14-35	39			x		3 E-38	Cystolic regulator *Pianissimo*	0
CA-GT	9-14-36	40	x		x	x	*	No significant match	
CA-GT	9-14-37	41	x	x			4 E-14	Fad dependent oxidoreductase	1 E-38
CA-GT	9-14-38	42	x	x			*	No significant match	
CA-GT	9-14-39	43		x			2 E-33	No significant match	
CA-GT	9-14-40	44			x		2 E-29	No significant match	
CT-GA	10-13-19	45				x	*	No significant match	
CT-GA	10-13-20	46		x			*	No significant match	
CT-GT	10-14-14	47	X	x	x		8 E-19	Hypothetical fungal proteins	4 E-74
CT-GT	10-14-15	48	X	x	x		8 E-16	Hypothetical fungal protins	4 E-74
CT-GT	10-14-17	49			x	x	*	No significant match	
CT-GG	10-16-7	50				x	1 E-12	No significant match	
CT-GG	10-16-9	51				x	*	No significant match	
CT-GG	10-16-10	52	x				*	No significant match	
CT-GG	10-16-15	53	x				*	No significant match	
CC-AG	11-4-1	54		x	x		*	No significant match	
CC-AG	11-4-2	55	x				*	No significant match	
CC-AG	11-4-6	56		x	x		*	No significant match	
CC-GA	11-13-10	57			x		3 E-83	No significant match	

Primers[a]	Band Id.[b]	TDF No.	Stage[c] 1	2	3	4	E-value versus cDNA library[d]	Annotation[e]	E-value NCBI[f]
CC-GA	11-13-11	58	x				E E-92	Spermine/spermidine synthase	1 E-136
CC-GA	11-13-13	59				x	2 E-6	No significant match	
CC-GA	11-13-14	60	x		x		9 E-61	GP1 Anchor Protein	1 E-37
CC-GA	11-13-15	61	x				2 E-61	GP1 Anchor Protein	1 E-37
CC-GA	11-13-17	62	X	x	x		2 E-65	GP1 Anchor Protein	1 E-37
CC-GA	11-13-18	63			x		2 E-65	Reverse transcriptase	7 E-15
CC-GA	11-13-20	64				x	4 E-44	Acetyl coA synthase	1 E-52
CC-GA	11-13-21	65		x	x		3 E-48	DUF221 Protein	0
CC-GA	11-13-22	66		x	x		3 E-38	DUF221 Protein	0
CC-GA	11-13-28	67	X	x			1 E-37	ATP Synthase	1 E-14
CC-GA	11-13-32	68		x			1 E-17	Reverse transcriptase	7 E-5
CC-GT	11-14-3	69			x		2 E-11	Glycine dehydrogenase	2 E-125
CC-GT	11-14-6	70		x	x		*	No significant match	
CC-GT	11-14-18	71	X	x	x	X	*	No significant match	
CC-GT	11-14-19	72	x				*	No significant match	
CC-GT	11-14-20	73	x			x	*	No significant match	
CC-GT	11-14-21	74	x			x	*	No significant match	
CC-GT	11-14-22	75	x			x	*	No significant match	
CC-GT	11-14-23	76	x			x	*	No significant match	
CG-GA	12-13-6	77				x	9 E-69	No significant match	
CG-GA	12-13-13	78	x	x	x		9 E-39	Serine/threonine protein kinase	1 E-171
CG-GA	12-13-15	79	x			x	1 E-38	Calcium permease	1 E-73
CG-GA	12-13-16	80	x			x	*	No significant match	
CG-GA	12-13-18	81	x				1 E-43	C2H2 Transcription factor	6 E-32
CG-GT	12-14-7	82	x	x	X		*	No significant match	
CG-GG	12-16-2	83	x	x	x		1 E-88	Glycine dehydrogenase	2 E-125
CG-GG	12-16-4	84			x		*	No significant match	
CG-GG	12-16-5	85	x				1 E-44	No significant match	
CG-GG	12-16-6	86		x			6 E-23	Pol Polyprotein	5 E-76

Primers[a]	Band Id.[b]	TDF No.	Stage[c] 1	2	3	4	E-value versus cDNA library[d]	Annotation[e]	E-value NCBI[f]
GA-AG	13-4-12	87		x			*	No significant match	
GA-AG	13-4-14	88				x	*	No significant match	
GA-AG	13-4-15	89	x				2 E-7	Glycine dehydrogenase	2 E-125
GA-TG	13-8-4	90	x				*	No significant match	
GA-TG	13-8-14	91		x			6 E-40	Heat shock protein 70	0
GA-TG	13-8-15	92		x		x	5 E-53	Cell cycle control proteins	0
GA-TG	13-8-16	93				x	*	No significant match	
GA-GA	13-13-1	94				x	*	Hypothetical fungal proteins	4 E-8
GA-GA	13-13-3	95		x			1 E-179	Integral membrane protein/Pth11	6 E-64
GA-GA	13-13-4	96				x	1 E-17	No significant match	
GA-GA	13-13-5	97			x		4 E-14	Hypothetical fungal protein	3 E-30
GA-GA	13-13-6	98	x	x			*	No significant match	
GA-GA	13-13-10	99	x	x			2 E-11	Coatomer subunit alpha	2 E-116
GA-GA	13-13-12	100		x	x		3 E-35	Coatomer subunit alpha	2 E-116
GA-GT	13-14-14	101				x	1 E-59	Caseine kinase	0
GA-GT	13-14-18	102				x	6 E-69	No significant match	
GA-GT	13-14-22	103				x	*	No significant match	
GA-GG	13-16-9	104			x		2 E-58	F Box domain protein	2 E-105
GA-GG	13-16-13	105				x	6 E-59	Autophagy related protein	7 E-152
GA-GG	13-16-14	106		x	x		4 E-63	Authophagy related protein	7 E-152
GA-GG	13-16-15	107	x				*	No significant match	
GA-GG	13-16-17	108	x				5 E-37	Acetolactase synthase	0
GA-GG	13-16-18	109	x	x	x		5 E-34	Pap2 domain protein	1 E-72
GT-AG	14-4-3	110				x	6 E-8	Glycine dehydrogenase	2 E-125
GT-AG	14-4-5	111				x	*	No significant match	
GT-AG	14-4-8	112		x	X		2 E-46	No significant match	
GT-AG	14-4-9	113	x				9 E-46	Hypothetical fungal proteins	8 E-28
GT-AG	14-4-16	114				x	2 E-10	Glycine dehydrogenase	2 E-125
GT-AG	14-4-18	115				x	5 E-27	C2H2 Transcription Factor RfeC	4 E-25

Primers[a]	Band Id.[b]	TDF No.	Stage[c] 1	2	3	4	E-value versus cDNA library[d]	Annotation[e]	E-value NCBI[f]
GT-AG	14-4-23	116	x	x		X	*	No significant match	
GT-AG	14-4-28	117	x	x			*	No significant match	
GT-TA	14-5-1	118	x				*	MFS monocarboxylic acid transporter	5 E-7
GT-TT	14-6-8	119		x	x	x	5 E-47	Mads Box Transcription Factor	7 E-46
GT-CT	14-10-1	120		x			*	No significant match	
GT-CT	14-10-3	121	x				*	No significant match	
GT-CC	14-11-3	122			x		*	No significant match	
GT-GA	14-13-1	123			x		*	No significant match	
GT-GA	14-13-2	124	x				*	No significant match	
GT-GA	14-13-3	125			x		*	No significant match	
GT-GA	14-13-9	126	x	x			4 E-89	C2H2 Zinc finger domain	0
GT-GA	14-13-10	127	x	x			*	No significant match	
GT-GA	14-13-11	128	x		x		5 E-48	AI Hook motif	3 E-26
GT-GA	14-13-12	129	x	x			5 E-7	Caseine kinase	0
GT-GA	14-13-17	130	x	x			9 E-12	No significant match	
GT-GA	14-13-19	131		x			*	No significant match	
GT-GT	14-14-1	132				x	9 E-46	Hypothetical fungal proteins	5 E-12
GT-GT	14-14-2	133	x				*	Phosphoglucomutase	4 E-9
GT-GT	14-14-3	134	x				*	Hypothetical fungal proteins	6 E-11
GT-GT	14-14-6	135				x	*	No significant match	
GT-GT	14-14-7	136				x	4 E-6	Hypothetical fungal proteins	8 E-82
GT-GT	14-14-9	137	x	x			*	No significant match	
GT-GT	14-14-10	138	x	x			4 E-53	Fuzzy related proteins	0
GT-GT	14-14-11	139		x	x	x	7 E-65	Gal4 like transcriptional activator	4 E-117
GT-GT	14-14-12	140			x	x	3 E-40	Integral membrane channel protein	8 E-66
GT-GT	14-14-13	141				x	2 E-38	Sac3/GanP domain protein	3 E-77
GT-GT	14-14-14	142				x	2 E-27	No significant match	
GT-GT	14-14-15	143				x	2 E-56	Gamma tubulin	6 E-76
GT-GT	14-14-16	144				x	2 E-58	Gamma tubulin	6 E-76

Primers[a]	Band Id.[b]	TDF No.	Stage[c] 1	2	3	4	E-value versus cDNA library[d]	Annotation[e]	E-value NCBI[f]
GT-GT	14-14-19	145	X	x	x		*	*Vitis vinifera* contig	3 E-11
GT-GC	14-15-2	146	x				*	No significant match	
GT-GC	14-15-7	148	x				*	No significant match	
GT-GC	14-15-9	149	x	x			*	No significant match	
GT-GC	14-15-11	150		x		x	*	Hypothetical fungal proteins	9 E-14
GT-GC	14-15-12	151		x		x	*	Hypothetical fungal proteins	7 E-11
GT-GC	14-15-13	152			x		*	No significant match	
GT-GC	14-15-14	153			x		*	No significant match	
GT-GG	14-16-8	154				x	*	No significant match	
GT-GG	14-16-12	155			x		1 E-53	Ring finger domain protein	1 E-11
GT-GG	14-16-13	156	x	x		x	2 E-23	No significant match	
GT-GG	14-16-14	157		x	x		3 E-19	Hypothetical fungal proteins	4 E-43
GT-GG	14-16-15	158			x		2 E-33	No significant match	
GT-GG	14-16-16	159		x			3 E-6	Reverse transcriptase	2 E-14
GT-GG	14-16-17	160			x		6 E-22	Reverse transcriptase	2 E-14
GT-GG	14-16-18	161	x				2 E-14	AT Hook domain	3 E-26
GT-GG	14-16-19	162	x	x	x		4 E-34	Vacuolar ATP Synthase	0
GC-AG	15-4-5	163	X	x	x	X	1 E-47	D-3 phosphoglycerate dehydrogenase	4 E-162
GC-AG	15-4-6	164				x	9 E-13	No significant match	
GC-AG	15-4-11	165				x	*	No significant match	
GC-AG	15-4-12	166				x	*	No significant match	
GC-TG	15-8-4	167			x		*	No significant match	
GC-TG	15-8-13	168			x		*	No significant match	
GC-CC	15-11-2	169	X	X		x	5 E-36	Hypothetical fungal proteins	2 E-141
GC-CC	15-11-4	170				x	8 E-38	Glycine dehydrogenase	2 E-125
GC-CC	15-11-5	171				x	*	No significant match	
GC-CG	15-12-5	172		x			7 E-23	Glycine dehydrogenase	2 E-125
GC-GA	15-13-4	173		x			*	No significant match	
GC-GA	15-13-6	174		x	x		3 E-57	No significant match	

Primers[a]	Band Id.[b]	TDF No.	Stage[c] 1	2	3	4	E-value versus cDNA library[d]	Annotation[e]	E-value NCBI[f]
GC-GA	15-13-7	175	x	x	X		5 E-39	Hypothetical fungal proteins	1 E-54
GC-GA	15-13-8	176	X	x	x	X	*	No significant match	
GC-GA	15-13-10	177	x	x	x		2 E-41	CobW Domain Protein	3 E-170
GC-GA	15-13-12	178		x			7 E-29	Hypothetical fungal proteins	2 E-141
GC-GA	15-13-14	179	x	x			1 E-17	Importin Beta-3 subunit	0
GC-GA	15-13-15	180		x			*	No significant match	
GC-GT	15-14-1	181	x				2 E-35	Glycine dehydrogenase	2 E-125
GC-GT	15-14-2	182	x	x	X	X	3 E-37	Glycine dehydrogenase	2 E-125
GC-GT	15-14-9	183			x		1 E-19	Hypothetical fungal proteins	1 E-167
GC-GT	15-14-10	184	x	x			3 E-30	No significant match	
GC-GT	15-14-11	185			x		6 E-6	No significant match	
GC-GT	15-14-13	186		x			1 E-37	No significant match	
GC-GT	15-14-14	187		x			*	*Oryza sativa* cDNA clone	7 E-27
GC-GT	15-14-23	188				x	2 E-61	No significant match	
GC-GT	15-14-25	189				x	4 E-20	No significant match	
GC-GT	15-14-26	190		x			1 E-37	DUF89 protein	4 E-177
GC-GT	15-14-27	191	x				*	No significant match	
GC-GT	15-14-28	192				x	2 E-58	No significant match	
GC-GT	15-14-29	193	x				2 E-39	No significant match	
GC-GT	15-14-30	194			x		8 E-52	No significant match	
GC-GT	15-14-31	195		x			5 E-6	Transcriptional activator Spt7	3 E-51
GC-GT	15-14-33	196	x	x			3 E-25	Chitin Synthase	0
GC-GC	15-15-13	197	x				9 E-46	Hypothetical fungal proteins	5 E-12
GC-GC	15-15-14	198				x	*	No significant match	
GC-GC	15-15-20	199				x	*	No significant match	
GC-GC	15-15-21	200		x			5 E-4	Hypothetical fungal proteins	1 E-58
GC-GC	15-15-22	201				x	2 E-40	Hypothetical fungal proteins	0
GC-GG	15-16-10	202	x	X	x		6 E-51	Histone transcriptional regulator	2 E-34
GC-GG	15-16-11	203	x	X	x		1 E-20	Histone transcriptional regulator	2 E-34

Primers[a]	Band Id.[b]	TDF No.	Stage[c] 1	2	3	4	E-value versus cDNA library[d]	Annotation[e]	E-value NCBI[f]
GC-GG	15-16-12	204	x	x	x		1 E-26	Hypothetical fungal proteins	3 E-87
GC-GG	15-16-16	205	x		x		1 E-10	Hypothetical fungal proteins	2 E-38
GC-GG	15-16-19	206	x	x		x	*	No significant match	
GC-GG	15-16-24	207		x		x	5 E-37	No significant match	
GG-AG	16-4-2	208	x			x	3 E-56	FlbD	1 E-4
GG-AG	16-4-3	209	x			x	4 E-40	Calcium binding proteins	1 E-61
GG-AG	16-4-5	210				x	7 E-26	Glycine dehydrogenase	2 E-125
GG-AG	16-4-7	211		x	x	X	1 E-66	Protoheme IX farnesyl transferase	3 E-72
GG-AG	16-4-8	212		x			7 E-56	WD Repeat Proteins	7 E-20
GG-AG	16-4-15	213			x		9 E-6	Hypothetical fungal proteins	8 E-22
GG-AG	16-4-16	214	X	x	x	X	3 E-6	No significant match	
GG-TG	16-8-8	215	x			x	3 E-64	PRELI/MSF1 domain protein	2 E-57
GG-TG	16-8-4	216		x			9 E-42	Hypothetical fungal proteins	2 E-41
GG-TG	16-8-7	217				x	*	No significant match	
GG-TG	16-8-9	218		x	x		4 E-32	Ring finger domain protein	7 E-130
GG-GT	16-14-14	219	x				3 E-9	Hypothetical fungal proteins	2 E-10
GG-GT	16-14-15	220		x			*	No significant match	
GG-GT	16-14-16	221		x			*	No significant match	
GG-GC	16-15-1	222		x		x	*	No significant match	
GG-GC	16-15-3	223				x	5 E-24	Hypothetical fungal proteins	0
GG-GC	16-15-8	224			x		1 E-12	No significant match	
GG-GC	16-15-11	225			x	x	1 E-47	Alcohol dehydrogenase	8 E-47
GG-GG	16-16-7	226		x		x	3 E-38	Transcriptional elongation factor Spt6	0
GG-GG	16-16-8	227	x			x	4 E-10	No significant match	
GG-GG	16-16-10	228	x				4 E-47	No significant match	
GG-GG	16-16-11	229				x	8 E-18	Hypothetical fungal proteins	3 E-30
GG-GG	16-16-15	230		x	x		*	No significant match	
GG-GG	16-16-21	231	X	x	x		3 E-23	Fatty aldehyde dehydrogenase	3 E-165

[a] Primer combinations are given by selective nucleotides in the following 5'-3' format: *Ase*I primer-*Taq*I primer.
[b] Each TDF Band was given a number based on the primers used (first two numbers) and the number band within each primer combination.
[c] The stages of expression are represented as follows: 1) pre-sporulation, 2) conidiophore initiation, 3) full sporulation, and 4) development of mature ascocarps. An x in each column indicates that the sequence was expressed in this stage. Where a sequence was expressed at multiple stages, an X in each column indicates that expression was higher in this stage than others within this band.
[d] TDFs with no match to the cDNA library are scored with a * in the blast search against the E. necator cDNA library. The E-value given is for the highest match against the cDNA library.
[e] Tblastx was conducted for each cDNA contig sequence against the NCBI database, and the annotation of the top match is given here. Where no contig was matched, the annotation is given on the direct search of the original TDF against the NCBI database.
[f] The E-values of the match are listed for the direct search of the cDNA library contig or TDF sequence against the NCBI database.

APPENDIX TWO: ADDITIONAL EXPERIMENTS

During the investigations that led up to the production of this work, several lines of investigation were pursued without useful result. In particular, the series of experiments designed to physically isolate the putative chemical signals for initiation and repression of sporulation. What follows is a brief description of the protocols followed and the general results observed.

Series One: Attempts to transfer the signal from one colony to another

Initially, attempts were made to simply transfer an initiating or repressing signal from a source colony to a target colony. For the initiating signal, colonies undergoing asexual sporulation were used as source colonies and colonies in pre-sporulation (i.e. in a purely vegetative state) were used as the target colonies. For the source colonies, mildew-free seedling leaves were inoculated with a conidial suspension of approximately 200 conidia/5 µl drop. Five days later, target colonies were grown by inoculating leaves under the same protocol. At the time of the experiment, source colonies were 8 days old and target colonies three. Connections between source and target colonies were attempted in a number of ways.

In initial experiments, both target and source colonies were inoculated on leaves on intact seedlings. The target and source colony were visually aligned and pressed together. A clamp was made by wrapping the ends of two popsicle sticks with cotton and binding the opposite end with an O-ring. These clamps were used to clamp together the two leaves once the colonies had been aligned. The colonies were allowed to remain that way for 24 hours and then the target colony was observed for signs of sporulation as compared to an unaltered control.

In later experiments, both target and source colonies were inoculated on surface sterilized powdery mildew-free leaves that had been placed on 1% water agar. Discs containing the 8-day-old source colonies were excised using cork borers. These colonies were placed upside down on the 3-day-old target colonies (i.e. so the top of the source

colony was in direct contact with the target colony). A piece of water agar was placed on top of the source-disk to weigh it down and provide better contact between colonies. This was left for 24-hours and observed for signs of sporulation relative to the unaltered control.

Additional attempts at transferring a signal were done in a similar manner to identify the repressing signal. In those experiments, mating colonies at approximately three weeks after inoculation were used as targets and sporulating colonies 8 days post-inoculation were used as targets. The target colony was observed for signs of repression of sporulation. Additional targets were colonies at 3 dpi. These were observed to see if colonies failed to initiate sporulation. Finally. colonies of one mating type at 8 dpi were applied to colonies of the opposite mating type at 3 dpi to see if contact between opposite mating types resulted in some visible phenotype.

Results

No changes in either initiation or repression of sporulation were observed in any target colony relative to the unaltered control. This could be due to a number of factors. First, the signals may not exist in any concentration outside of fungal hyphae. Secondly, good contact may not have been achieved between colonies. Finally, in experiments where a water agar plug was used to weigh down source colonies, the moisture from the water agar appeared to saturate the source colony and may have diffused the potential signal.

Series Two: Attempts at physical isolation of initiation signals

In this series of experiments, generally four flats of mildew-free seedlings were spray inoculated with isolate 10-18 and allowed to grow for 8 days. Five days after inoculation of source colonies, target colonies were inoculated. Young mildew-free grape seedling leaves were excised, surface sterilized and placed on water agar. Target colonies were inoculated using conidial suspensions of approximately 200 conidia per drop.

To isolate the chemical signal, heavily infected leaves were picked from the flats that had been spray inoculated 8 days previously. Generally 15-20 grams of infected leaves were harvested. Forty milliliters of the selected solvent (water, ethyl acetate, ethanol or methanol) was applied to the infected leaves and hand-shaken for five minutes.

The leaves were then removed from the solvent. In all experiments using water and in the initial experiment using ethyl acetate as a solvent, the solvent was then aliquoted into 1.5 ml Eppendorf tubes and placed in the speed vac. The speed vac was run for 30 minutes and then the remaining liquid in each eppendorf tube was combined with the liquid from one other, resulting in halving the initial number of eppendorf tubes. The procedure of running the speed vac for 30 minutes followed by a halving of the number of eppendorf tubes was repeated until all remaining solvent was in one remaining eppendorf tube. This was run in the speed vac until it was concentrated to a final volume of approximately 300 µl, a process which generally took an additional 60-90 minutes in the speed vac.

For later extracts using ethyl acetate, and all extracts using ethanol or methanol, the initial 40 ml volume of extract was concentrated down to approximately 1 ml using a roto-vap as it was thought the evaporating vapors from these experiments would damage the speed vac.

The resulting concentrate was generally dark green and oily. Where water was used as a solvent, the extract was applied directly to target colonies. Where toxic solvents such as ethyl acetate, ethanol or methanol were used, the extract was diluted by 10 times using sterile water. Ten µl drops of the extract were placed on the target colonies and allowed to dry. Control colonies of two types were used. In the first, the colonies received a ten times dilution of the pure solvent to assess what effect the solvent itself had on colony development. The second control received no treatment.

Results

Results from these experiments were ambiguous at best. In the first experiment involving ethyl acetate, the majority of the colonies died. Three did survive and sporulated 12-24 hours in advance of untreated controls. A repeat of the ethyl acetate experiment using the roto-vap instead of the speed vac to concentrate the solvent resulted in the death of all treated colonies. The colonies appeared to shrivel and in some instances the leaf underneath the colony started to die. In some cases where the colony was larger than the droplet, the margin of the colony survived but showed signs of stress like aerial hyphae and severely reduced growth as compared to untreated controls.

Results from the ethanol and methanol experiments followed what was seen in the second ethyl acetate experiment. This could be due to a number of factors. Ethyl acetate and to a lesser extent ethanol and methanol showed a deleterious effect on the colonies even at a ten-times dilution. Colonies which received the solvent-dilution control themselves showed signs of stress, although not to the extent of the colonies receiving the leaf extract. In addition, the extract droplets applied to the treatment colonies never dried. It could be that colonies were simply suffocated.

The water extracts did not exhibit this toxic effect. They did dry completely, albeit a bit slower than the control plain water droplets. The colonies occasionally exhibited a few signs of stress such as aerial hyphae or thicker, ropier hyphae on the colony surface. These were results occasionally observed in untreated controls however and were not limited to the water extracts. Although the water extracts did not kill the treated colonies, neither did they appear to have any effect on timing of sporulation.

Discussions with Dr. Donna Gibson indicated that this method of searching for the initiating signal may not prove fruitful. It will likely be impossible to grow sufficient quantities of fungus for extraction. Even inoculating four colonies of vigorous healthy seedlings produced at most 20 g of infected leaves and likely less than a gram of actual fungal tissue. In addition, the near impossibility of separating the fungus from the leaf

surface means that any extraction of the fungus will necessarily involve extracting compounds from the host, some of which are almost undoubtedly harmful to the fungus.

Dr. Gibson suggests an alternative approach. Should genes be identified with critical functions in sporulation, an extract could be made from an isolate where one or more of these genes were temporarily silenced. This could be compared with an extract from a wild type fungus. The chemical profiles of both could be compared, and the signal compound identified based on what was present in the wild type but lacking in the mutant.