ILLUMINATING HOW LIGHT AFFECTS THE WHEAT FUNGAL PATHOGEN *ZYMOSEPTORIA TRITICI*

by

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Dedicated to my husband, Phil. I couldn't have done this without you.

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LIST OF ABBREVIATIONS

(6-4)PP –	6-4 pyrimidine-pyrimidone
AER –	Alternative Excision Repair
CPD –	Cyclobutane Pyrimidine Dimer
Cry –	Cryptochrome
Cry-DASH –	Cryptochrome-(*Drosophila, Arabadopsis, Synechocystis*, Human)
DHN –	Dihydroxynapthalene
DMI –	Demethylation Inhibitors
Dpi –	Days Post Inoculation
FAD –	Flavin Adenine Dinucleotide
Frq –	Frequency
GO –	Gene Ontology
Hph –	Hygromycin Phosphotransferase
KOG –	EuKaryotic Orthologous Group
LOV –	Light, Oxygen, Voltage
MAPK –	Mitogen-Activated Protein Kinase
NER –	Nucleotide Excision Repair
NOG –	Non-orthologous Group
NRPS –	Non-ribosomal Peptide Synthetases
NRPS-L –	NRPS-like
ORP –	Opsin Related Protein
PAS –	Per-Arnt-Sim
Per –	Period Circadian Protein
Arnt –	Aryl Hydrocarbon Receptor Nuclear Translocator
Sim –	Single Minded
PCWDE –	Plant Cell Wall Degrading Enzymes
PDA –	Potato Dextrose Agar
Phr -	Photolyase
Phy –	Phytochrome
PKS –	Polyketide Synthase

PKS-L –	PKS-like
QoI –	Quinine Outside Inhibitors
QTL –	Quantitative Trait Locus
ROS –	Reactive Oxygen Species
SAR –	Systemic Acquired Resistance
SDHI –	Succinate Dehydrogenase Inhibitors
STB –	Septoria tritici Blotch
SSP –	Small Secreted Protein
UV -	Ultraviolet
VVD -	VIVID
WCC –	White Collar Complex
WC1 –	White Collar 1
WC2 –	White Collar 2
YSA –	Yeast Sucrose Agar

ABSTRACT

Light is a critical part of the environment and can cause a drastic amount of damage in the fungal cell. Understanding how phytopathogens respond to and cope with light is important for knowing how to control the diseases they cause. Despite the importance of *Zymoseptoria tritici* as a fungal pathogen of wheat, little is known about the reaction of this fungus to light. To test for general light responses, cultures of *Z. tritici* were grown *in vitro* under white, blue or red light, and their transcriptomes were compared with each other and to those obtained from control cultures grown in darkness. There were major differences in gene expression between the dark compared to the individual light treatments, indicating that *Z. tritici* can sense and respond to light. Genes for effectors that have been shown previously to be involved in pathogenicity also were upregulated in one or more of the light treatments, suggesting a possible role of light for infection. To study how *Z. tritici* responds to light, the light-sensing protein ZtVVD was examined and a deletion mutant was generated. ZtVVD was shown to be an important gene for infection, but dispensable for most general growth and stress responses. The genome of *Z. tritici* showed a wide range of pathways for repairing the damage to DNA done by UV light. A phylogenetic analysis of the proteins in the photolyase/cryptochrome class of proteins revealed the presence of three photolyase proteins and one Cry-DASH cryptochrome. A deletion mutant of one photolyase, ZtPhr1, showed no developmental disruption or changes in stress response, indicating a wide range of redundancies in UV repair pathways, and the necessity of further studies in this area.

CHAPTER 1. INTRODUCTION

1.1 Why light?

Light is the source of most energy on this planet. Photosynthetic bacteria and plants take this energy, convert it to chemical energy, and are then consumed by other organisms who use that energy, and are sometimes eaten themselves. And yet, light can be detrimental, able to mutate DNA and form dangerous compounds, so organisms have evolved ways to cope and protect themselves from the damaging effects of light, while still reaping the benefits.

To a phytopathogenic fungus that lives on a plant's surface, light is a very important environmental cue. Light indicates photosynthesis is occurring, which means the stomata are open for invasion. It also means that dangerous UV radiation is present, which can damage cells. As such, phytopathogenic fungi have developed ways to sense and respond to light, including multiple mechanisms of preventing and repairing light-induced damage.

1.2 Dangers of light

UV light can damage an organism's DNA in at least two ways. The main type of damage induced by UV light to DNA is through the formation of cyclobutane pyrimidine dimers (CPDs). In this form of damage, two consecutive pyrimidines, thymine or cytosine, form a square bond that causes mutations and possibly death of the cell (Figure 1.1). Most commonly, these are thymine-thymine dimers, but any cytosine involved will be deaminated and turned into a thymine (Schuch and Menck 2010). Translesion polymerases commonly introduce an adenine nucleotide, so a thymine-thymine dimer is less likely to introduce a mutation than a cytosine CPD (Prakash and Prakash 2002). Another type of DNA damage caused by UV light is known as a 6-4 pyrimidine-pyrimidone [(6-4)PP], in which alent bond forms between the C6 in one ring and the C4 in the next (Figure 1.1). These form at a third of the rate of CPDs, but they tend to be more mutagenic. The third type of lesion is a Dewer isomer, which is formed from a (6-4)PP lesion that absorbs additional UV radiation (Figure 1.1) (Douki and Sage 2016).

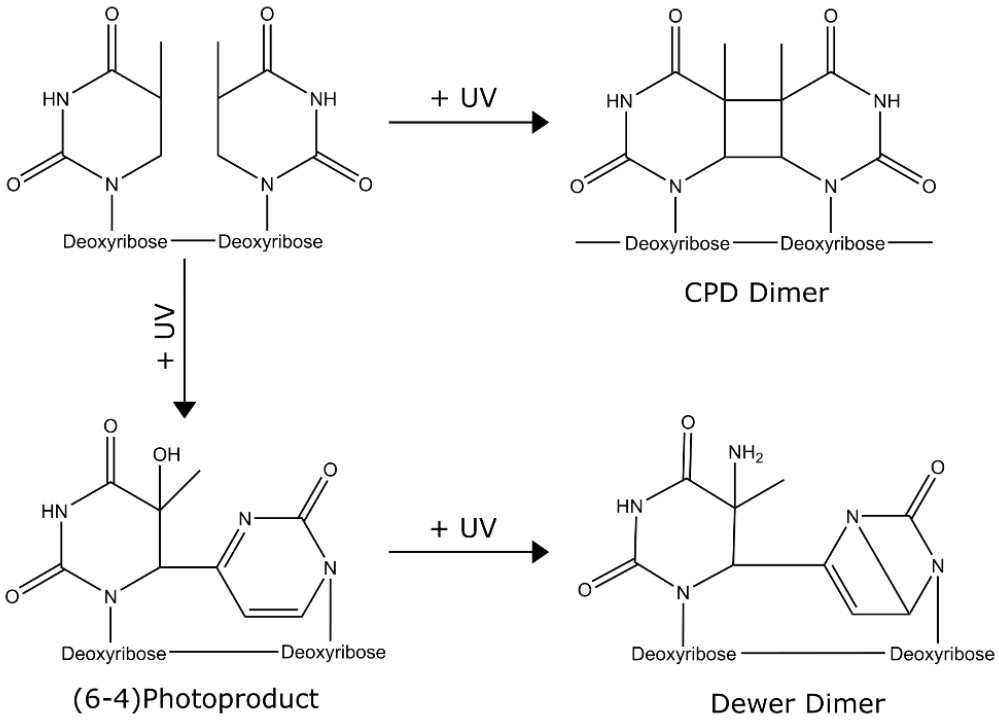


Figure 1.1 Diagram of the potential UV lesions formed in DNA, using thymine as the bases.

UV light also damages organisms by stimulating the production of reactive oxygen species (ROS) (Heck et al. 2003). These ROS include free radicals, compounds with an unpaired electron, derived from oxygen species such as superoxide (O_2^-) and hydroxyl radicals ($^{\bullet}OH$), as well as reactive oxygen species that are not free radicals, such as hydrogen peroxide (H_2O_2) (Aguirre et al. 2005). These ROS are formed not only in response to and directly by UV light; photosensitizers, often used as toxins, and cellular respiration can also form ROS (Heck et al. 2003; Daub et al. 2005; Beltrán-García et al. 2014; Sun et al. 2018). The generation of ROS in aerobic organisms is a critical part of respiration, but additional ROS produced by other factors can overwhelm the coping mechanisms and be harmful to cells.

1.3 How do phytopathogens sense light?

There are two primary ways that fungi prepare for the presence or absence of light: directly sensing its presence, and by predicting when light will come. Fungi have multiple proteins to

directly sense light, all of which use some form of chromophore, a compound that enters an excited state when activated by light. The shift to the excited state changes the conformation and structure of the protein. Once excited, the protein can activate or repress other genes, or do other tasks. Fungi can also anticipate when light will arrive via the circadian rhythm. In nature, the day:night light cycles are very predictable, as day lengths do not vary much from day to day.

Both the circadian rhythm and multiple light-sensing proteins have been well studied, mainly in model filamentous fungi such as *N. crassa* and species in the genus Aspergillus, such as *A. nidulans* and *A. fumigatus*. Interest in the effects of light in the model fungi has increased due to the potential to use these mechanisms for control of fungal growth and development, and these genes for sensing or responding to light are being analyzed in other species of fungi as well. These two methods of sensing or anticipating light can be very tightly interwoven in the species that have both, as not all fungal species utilize the circadian rhythm or have any proteins to sense light directly.

1.4 Light-Sensing Proteins

Fungi are known to detect at least two spectral ranges of light: blue and red. Detection of each color has potential advantages to the fungus. Blue light is closest to UV, and often coincides with the presence of UV light in nature; as such, there are multiple proteins and methods that fungi use to detect blue light. Red is often the first light that can be sensed at the beginning of the day and can be used to prepare for the approach of day. Red light would also be prominent near sunset where it could signal the transition from day to night. Green light is in the middle of the light spectrum, however the phyllosphere inside of a plant is exposed primarily to green light, as the other wavelengths are absorbed by chlorophyll, and the green is transmitted (Alsanius et al. 2019). As such, phytopathogens should be able to benefit from an ability to sense green light.

The light-sensing proteins in general are very well conserved through fungal lineages but have a variable evolutionary history. Some proteins are more widespread and ancient than others, present in a wider variety of fungal lineages, while others are more restricted (Figure 1.2). Despite this, the genes themselves are very well conserved within the lineages when present.

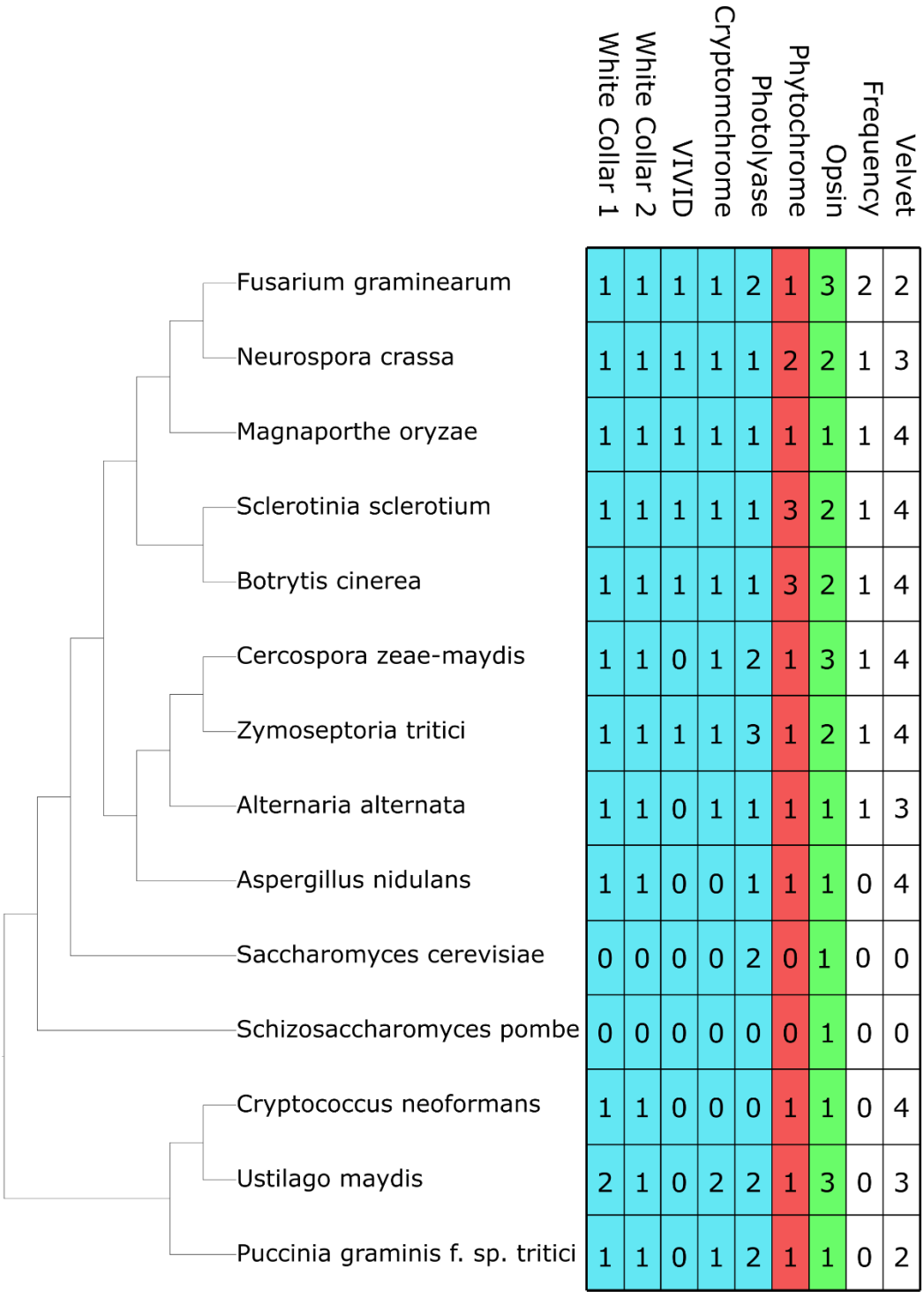


Figure 1.2 A phylogenetic table showing the presence or absence of major light -sensing and -responsive genes in a variety of fungi in Dikarya, generated using known phylogenetic relationships. The colored backgrounds indicate the color light sensed by these proteins. Presence was determined using blastn and tblastn against genome assemblies present in NCBI.

1.5 The White Collar Complex: White Collar 1 and White Collar 2

The primary light-sensing protein apparatus in fungi is the White Collar Complex (WCC), formed by the proteins White Collar 1 (WC1) and White Collar 2 (WC2). These proteins were originally described in *Neurospora crassa* (Harding and Turner 1981; Degli-Innocenti and Russo 1984; Ballario et al. 1996; Linden and Macino 1997), but have been found in all fungal families. Early-branching fungi such as Zygomycetes tend to have multiple WC1 and WC2 genes, while Ascomycetes and Basidiomycetes usually have zero to one of each (Figure 1.2) (Idnurm et al. 2010; Rodriguez-Romero et al. 2010). Of the two proteins in the WCC, WC1 has been studied the most, as it is the primary light sensor driving light responses.

Both WC1 and WC2 contain PAS domains (Per-Arnt-Sim: Period Circadian Protein – Aryl Hydrocarbon Receptor Nuclear Translocator – Single Minded) (Figure 1.3). The PAS domains are sensing domains found in proteins that often function by binding to another protein or molecule. In the case of the WCC, the PAS domains facilitate the dimerization of the two component proteins. LOV (Light, Oxygen, Voltage) domains are a subset of PAS. The LOV domain in WC1 acts as the primary light-sensing component and is the site where the FAD (flavin adenine dinucleotide) chromophore is bound to facilitate the sensing of light (Figure 1.3, 1.4). The third important type of domains in these proteins are zinc fingers (Figure 1.3). These domains allow for DNA binding so that the WCC can act as a transcription factor.

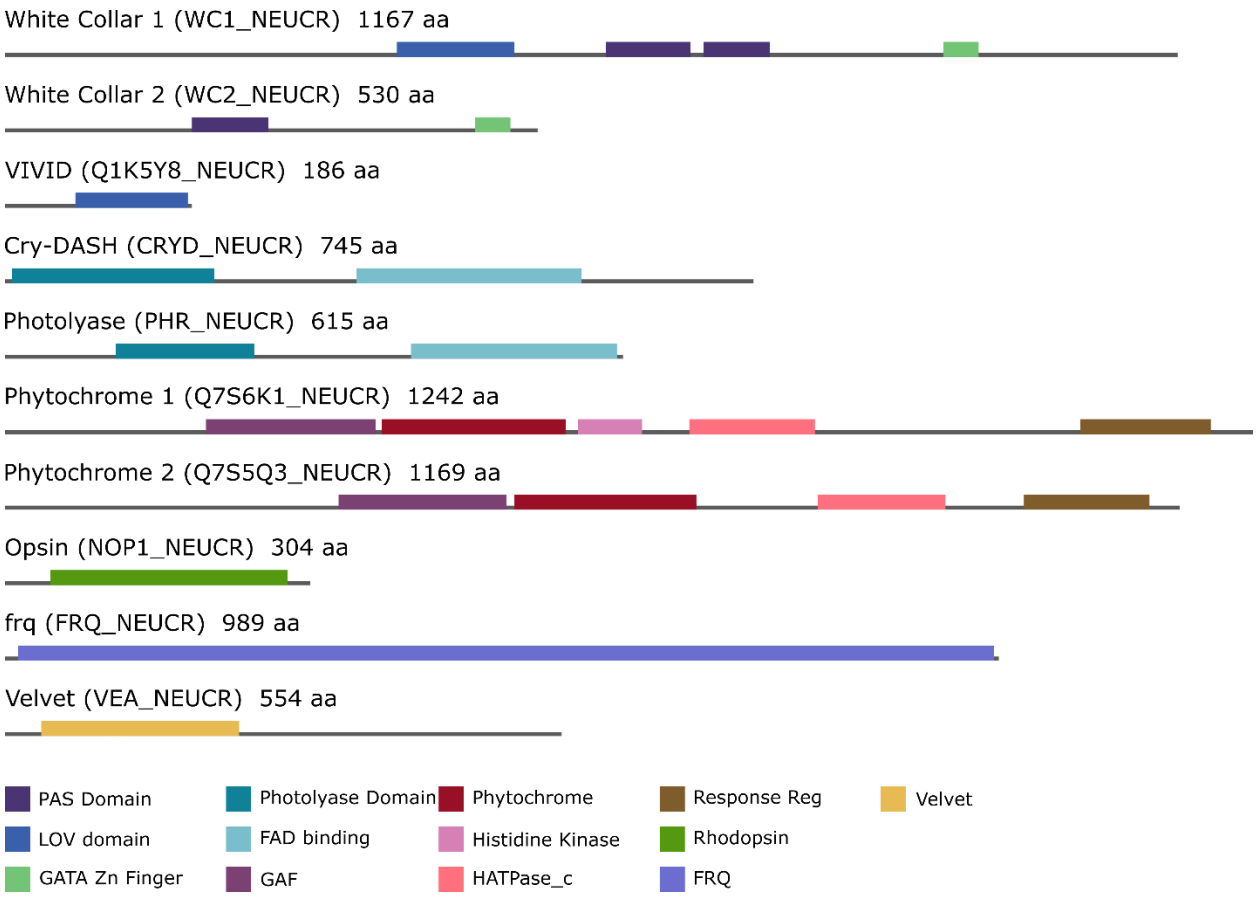


Figure 1.3 A listing of the major light-sensing and -responsive genes from *Neurospora crassa* and the encoded domains. Information was gathered from the Pfam and SMART domain databases (Letunic et al. 2015; Letunic and Bork 2018; El-Gebali et al. 2019).

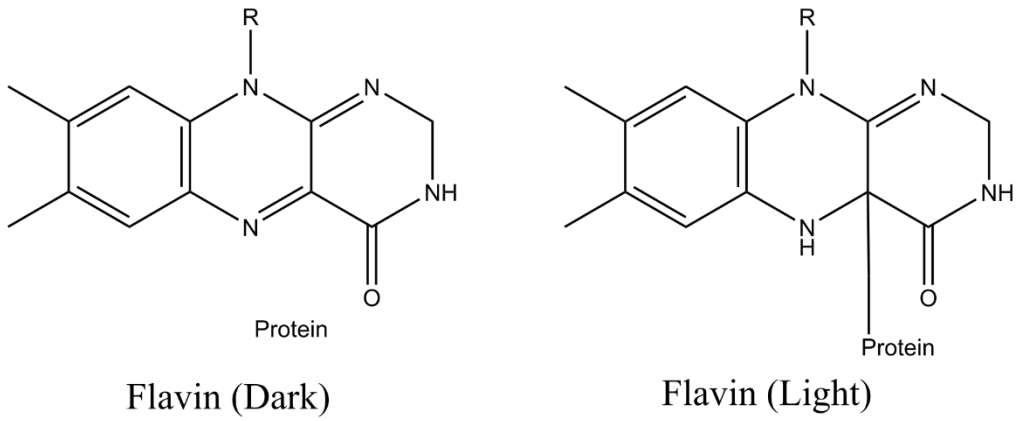


Figure 1.4 The structure of a flavin chromophore before and after exposure to light. The R represents an adenine dinucleotide.

The WCC is involved in controlling many aspects of fungal cell growth, development, and reproduction (Degli-Innocenti and Russo 1984; Linden and Macino 1997; He et al. 2002; Linden 2002; Lewis et al. 2002; Ambra et al. 2004; Casas-Flores et al. 2004; Lu et al. 2005; Esquivel-Naranjo and Herrera-Estrella 2007; Kihara et al. 2007; Estrada and Avalos 2008; Ruiz-Roldán et al. 2008; Purschwitz et al. 2009; Kim et al. 2011a, c; Canessa et al. 2013; Fuller et al. 2013; Yang et al. 2013b; Ohm et al. 2013; Yang and Dong 2014; Pruß et al. 2014; Kim et al. 2015b; Yang et al. 2016; Kumar et al. 2017; Shakya and Idnurm 2017; Xu et al. 2017; Krobanan et al. 2019; Tang et al. 2020). Disruption of its component genes can affect many systems in the cell; however, mutations affecting the WCC are not known to be lethal, as the fungi can still grow and function, but often with abnormalities in their abilities to sense and respond to light.

1.5.1 VIVID

VIVID (VVD) is the second major blue-light-sensing protein in fungi. The primary function of this protein is photoadaptation, in which the fungus adapts to the amount of light, and thus can differentiate between light intensities and ignore low levels of illumination such as moonlight (Schwerdtfeger and Linden 2003; Malzahn et al. 2010). This is done via a negative feedback loop, where the WCC induces production of VVD after light exposure, and VVD negatively regulates the action of the WCC (Malzahn et al. 2010; Chen et al. 2010; Hunt et al. 2010). Unlike the WCC, VVD is only found in the Ascomycota, and not in the other classes of fungi (Figure 1.2) (Corrochano 2019).

The existence of this protein was discovered after a mutant in *N. crassa* with a deep orange color in constant light, due to constitutive production of carotenoids was found (Shrode et al. 2001; Schwerdtfeger and Linden 2001; Chen and Loros 2009). This complete loss-of-function mutant was still able to sense and respond to light, but photoadaptation was impaired, with an inability to differentiate between high- and low-intensity light (Schwerdtfeger and Linden 2003). Like WC1, VVD contains a LOV domain that binds a FAD chromophore, and a PAS domain that allows for protein-protein interactions (Figure 1.3, 1.4).

VVD plays a role in multiple processes in fungi; however, it has not been studied in many species. The primary processes that VVD seems to affect are controlling pigmentation and reproduction, however it may be involved in additional processes that do not show obvious

phenotypes (Shrode et al. 2001; Seibel et al. 2012; Castrillo and Avalos 2014; Bazafkan et al. 2017; Schmoll 2018; Tong et al. 2018; Zhang et al. 2020).

The genome of the industrially important fungus *Trichoderma reesi* contains a related protein known as ENVOY (Schmoll et al. 2005). ENVOY provides the same function in *T. reesii* as VVD does in other fungi, being a negative regulator of the WCC, but it cannot directly complement VVD in *N. crassa* (Castellanos et al. 2010). ENVOY is important for full growth in the light and for fertility during sexual reproduction (Schmoll et al. 2005; Schmoll 2018).

1.5.2 The Cryptochrome/Photolyase Family

The other major class of blue light-sensing proteins is the cryptochrome/photolyase family. These proteins also use a FAD-based chromophore like the WCC and VVD proteins. Cryptochrome/photolyases are an ancient class of proteins, and can be found in every kingdom of life, from bacteria to animals such as humans (Daiyasu et al. 2004). There are two subclasses in the cryptochrome/photolyase protein family in fungi: photolyase and Cry-DASH (*Drosophila, Arabidopsis, Synechocystis*, Human). The primary distinction between these subclasses is that photolyase proteins work to repair damaged DNA, while the Cry-DASH proteins have lost that ability and instead act as signaling proteins. Both classes of proteins have similar structures, each with a photolyase or photolyase-like domain, and a binding domain for the FAD chromophore (Figure 1.3, 1.4).

Cry-DASH proteins are related to the photolyase proteins but do not contain a functional photolyase repair domain (Figure 1.3). These proteins diverged from the photolyases before the different kingdoms of life diverged, as evidenced by the variety of organisms in which they are found. Cry-DASH proteins act as blue-light regulators, unlike the photolyase proteins. There has been limited study of these proteins and their functions in fungal cells. While there have been some signs of Cry-DASH proteins having a regulatory role in fungal cells, they tend to be minor (Veluchamy and Rollins 2008; Froehlich et al. 2010; Castrillo et al. 2013; Cohrs and Schumacher 2017; Wang et al. 2017a). In some species, a Cry-DASH protein may function in regulating reproduction and the formation of reproductive structures, but it is not a necessary role in many of those species (Veluchamy and Rollins 2008; Castrillo et al. 2013; Cohrs and Schumacher 2017; Wang et al. 2017a). Similarly, they may be involved in regulating, but are not required for, secondary metabolism (Castrillo et al. 2013; Wang et al. 2017a). In some species, the expression

of Cry-DASH proteins and the WCC are linked (Froehlich et al. 2010; Castrillo et al. 2013; Cohrs and Schumacher 2017; Wang et al. 2017a). In most of these species, production of the Cry-DASH protein is induced by light in a WCC-dependent manner. However, in *Cordyceps militaris*, both the WCC and the Cry-DASH protein are required for expression of both (Froehlich et al. 2010; Castrillo et al. 2013; Cohrs and Schumacher 2017; Wang et al. 2017a).

The photolyase subclass of proteins repairs DNA using blue light and this function will be discussed in section 1.8.2, however some photolyase proteins also have rudimentary regulation functions. In *Trichoderma atroviridae*, *phr1* may regulate its own expression (Berrocal-Tito et al. 2007). In *Cercospora zeae-maydis*, knockout mutants of *phl1* show increased conidiation and decreased secondary metabolism (Bluhm and Dunkle 2008). Similarly, *cryA* in *Aspergillus nidulans* acts as a photolyase and a repressor of sexual reproduction (Bayram et al. 2008a).

1.5.3 Phytochrome

Phytochrome is a red and far-red light-sensing protein found throughout most kingdoms of life. They were originally found in plants, hence the name, but have since been found in bacteria and fungi (Duanmu 2014, Li 2015, Montgomery 2002). They use a heme-derived linear tetrapyrrole known as a bilin as a chromophore for sensing light, likely biliverdin (Figure 1.5) (Blumenstein et al. 2005; Froehlich et al. 2005). The bilin chromophore has two forms, one when it is exposed to red light (625 – 700 nm), and one when exposed to far red light (700 – 780 nm) (Figure 1.5) (Yu and Fischer 2018).

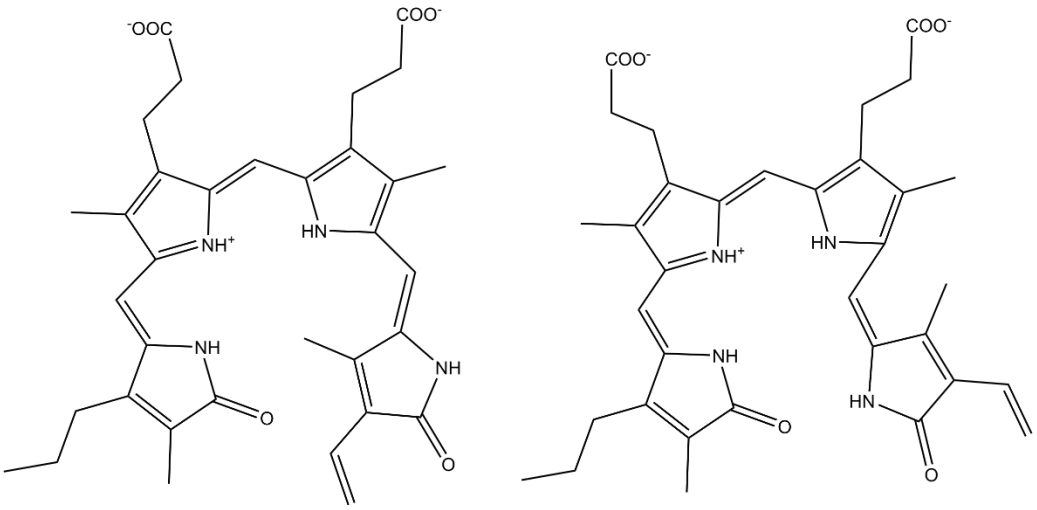


Figure 1.5 The structure of a biliverdin chromophore when exposed to red versus far-red light.

Fungal phytochromes resemble bacterial phytochromes more than those of plants (Blumenstein et al. 2005). The order of the various domains in the fungal phytochromes resembles that of those in bacteria. However, the fungal phytochromes regularly contain a response-regulator domain which is not commonly present in those of bacteria (Figure 1.3). Phytochromes also often contain conserved cysteines, one found in bacterial PAS domains, and another in plant small-ligand binding domains. Fungal phytochromes contain the bacterial conserved cysteine, but not the one found in plants (Blumenstein et al. 2005; Vierstra and Karniol 2005; Karniol et al. 2005).

One of the main functions of the phytochrome proteins in the fungal life cycle is regulating reproduction. Specifically, red light and the phytochromes control asexual development, such as conidial formation in *Aspergillus* spp. and *Alternaria alternata* (Fuller et al. 2013; Röhrig et al. 2013; Igbalajobi et al. 2019). Phytochromes can also regulate sexual reproduction. As with other aspects of fungal phtotobiology, light regulation with the phytochromes can be through induction or repression (Mooney and Yager 1990; Blumenstein et al. 2005; Sánchez-Arreguin et al. 2019). Phytochromes have also been shown to be involved in other stress responses. In two species, *Aspergillus nidulans* and *Alternaria alternata*, this occurs via the *Hog1*/*SakA* pathway, which regulates oxidative stress response (Idnurm and Bahn 2016; Igbalajobi et al. 2019).

1.5.4 Opsins

Fungal opsins are putative green-light sensors, found sporadically throughout fungi (Idnurm et al. 2010). Curiously, they are found more consistently among phytopathogens (Adam et al. 2018). These proteins are related to the animal rhodopsins (Type II), one of the proteins found in eyes (Ernst et al. 2014). However, they are more closely related to bacterial rhodopsins (Type I), which are light-driven proton pumps used for ion transport and for sensory input in the prokaryotic kingdoms *Bacteria* and *Archaea*, and which have recently been found in lower *Eukaryota*, such as fungi and algae, during the past 20 years (Bieszke et al. 1999b; Ernst et al. 2014).

There are four clades of opsin proteins in fungi, the *nop1*-like, CarO, opsin related proteins (ORPs), and Yro2 clades (Table 1.1) (Brown and Jung 2006; Wang et al. 2017b). The *nop1*-like and CarO opsins have a highly conserved lysine residue that binds a retinal chromophore via a Schiff base linkage, in which the retinal is converted to a protonated Schiff base and bound covalently to the conserved lysine (Figure 1.6) (Bieszke et al. 1999a). The other two clades, the ORPs and Yro2 opsins, do not contain this Schiff-base lysine. There are many other amino acid positions that are highly conserved in all four clades, so the ORPs and Yro2 clades of opsins without the retinal-binding lysine should still function, although their specific function is uncertain. The ORPs are found throughout the fungi, but characterization of knockout mutants has not elucidated their function (Brown 2004; Wang et al. 2017b). Proteins in the Yro2 class of opsins are involved in heat shock and coping with other stresses, such as oxidative, weak acid, or ethanol stress (Graul and Sadée 1997; Brown 2004; Stanley et al. 2010; Ghosh 2014; Takabatake et al. 2015).

Table 1.1 The clades of opsins, whether they bind with retinal, and their functions.

	Retinal-binding?	Function
nop1-like		
NR-like	Yes	Light sensor
LR-like	Yes	Light-driven proton pump
CarO	Yes	Light-driven proton pump
ORP	No	Unknown
Yro2	No	Used to tolerate stresses, primarily heat-shock

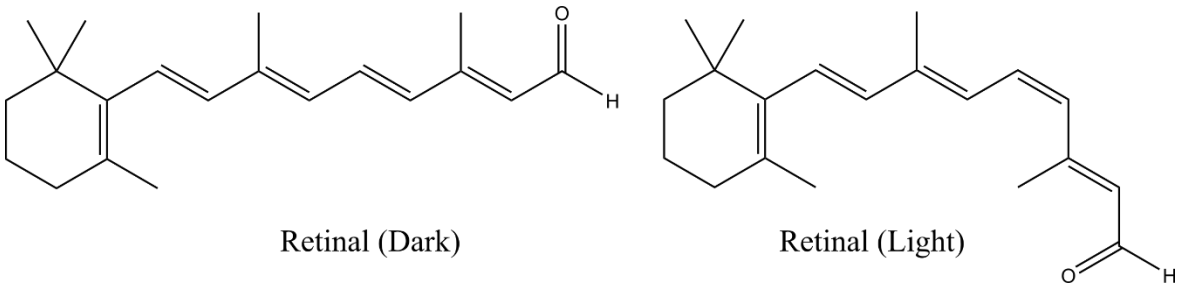


Figure 1.6 The structure of the retinal chromophore when present in the dark versus in light.

The two clades that can bind the retinal chromophore, *nop1*-like and CarO, will be the primary focus of this discussion. The *nop1*-like opsins, which are like the sensory rhodopsin II group in bacterial rhodopsins, can be further broken down into two subclasses. The *Neurospora crassa*-like receptors (NR-like) show no proton-pumping activity and seem to act primarily as light sensors (Brown et al. 2001; Adam et al. 2018). In contrast, the *Leptosphaeria maculans*-like receptors (LR-like) do act as light-driven proton pumps (Waschuk et al. 2005). The CarO-like opsins, or auxiliary opsin-related proteins, also act as light-driven proton pumps (Fan et al. 2011; Adam et al. 2018). These were found in the carotenoid biosynthesis cluster in *Fusarium fujikuroi* (Avalos and Estrada 2010).

Many of these proteins, regardless of their class, have little evidence demonstrating their function in the cell. Multiple opsin mutants have been characterized, but few have shown strong phenotypes, and many knockout mutants show no significant phenotypes at all (Bieszke et al. 1999a; Estrada and Avalos 2009; Heller et al. 2012). The primary functions attributed to opsins have involved stress tolerance, including fungicide and oxidative stresses (Temme et al. 2012; Lyu et al. 2016; Wang et al. 2017b). Opsin proteins have also been implicated in playing a major, but not critical, role in spore production and germination (Bieszke et al. 2007; Temme et al. 2012; García-Martínez et al. 2015; Lyu et al. 2016; Wang et al. 2017b). In some species, production of proteins by the various opsin genes is controlled by the WCC (Bieszke et al. 2007; Panzer et al. 2019).

1.6 Light-Regulated Proteins

While all proteins described previously are active in sensing light, the following proteins have different roles, but are still critical components of the light-response system.

1.6.1 Velvet Complex

Velvet is the primary protein complex downstream of the light-sensing proteins. Although none of these proteins actively senses light, they are among the light-responsive proteins (Bayram et al. 2008b, c; Yang et al. 2013a; Schumacher et al. 2015; Wang et al. 2019c). Thus far, this protein complex has been analyzed primarily among species in the genus *Aspergillus*. This protein complex consists of velvet A (veA), velvet B (velB), velvet C (velC) and viability of spores A (vosA), but also interacts with laeA, a regulator of many functions in fungi (Bayram et al. 2008b). The first of these proteins to be discovered was veA, when an *A. nidulans* mutant with a deletion in the N-terminal end of the protein showed abnormally high conidiation and low sexual reproduction, and no noticeable light responses (Käfer 1965).

The velvet complex, specifically veA, has been found to be important in many fungi, particularly with respect to growth and development. Strains with mutations in some members of the velvet complex, primarily veA, the main protein, show major growth defects. These include stunted or missing aerial hyphae, or defects in hyphal growth in general (Bayram et al. 2008b; Choi and Goodwin 2011; Merhej et al. 2012; Wang et al. 2019b; Tiley et al. 2019). VeA regulates sporulation in many species, but as is common in fungi, whether it represses or induces sporulation, both sexual and asexual, depends on the species (Kim et al. 2002; Calvo et al. 2004; Duran et al. 2007; Ni and Yu 2007; Bayram et al. 2008c; Duran et al. 2009; Merhej et al. 2012; Karakkat et al. 2013; Yang et al. 2013a; Kim et al. 2014b; Schumacher et al. 2015; Bazafkan et al. 2015; Rahnama et al. 2018; Wu et al. 2018; Zhang et al. 2018; Wang et al. 2019b; Tiley et al. 2019; Wang et al. 2019c). VeA also plays a role in secondary metabolism, including production of both toxins and pigments (Kato et al. 2003; Duran et al. 2007; Spröte and Brakhage 2007; Ni and Yu 2007; Bayram et al. 2008b; Kale et al. 2008; Duran et al. 2009; Choi and Goodwin 2011; Yang et al. 2013a; Schumacher et al. 2015; Zhang et al. 2018; Bayram et al. 2019). The velvet complex can also play a role in stress responses, primarily to oxidative stress, but it can affect osmotic stress responses in some fungi (Käfer 1965; Ni and Yu 2007; Yang et al. 2013a; Rahnama et al. 2018; Wu et al. 2018; Zhang et al. 2018; Wang et al. 2019b).

1.6.2 Circadian Rhythm - Frq

The circadian rhythm occurs in many forms of life to synchronize the stresses of the day with processes occurring in the organism. Circadian rhythms can be found throughout life (McClung 2006, Bell-Pederson 2005). While circadian rhythm is widespread throughout the kingdoms of life, the methods by which they are controlled can vary widely. Fungi are no different, possessing at least one unique method of controlling circadian rhythms.

The most well known and documented way of controlling circadian rhythm in fungi is by way of the *frq* gene, which is found primarily among Ascomycetes, and mostly within selected classes, including the Sordariomycetes, Dothideomycetes and Leotiomycetes; it is absent completely from the Saccharomycetes and almost completely absent from the Eurotiomycetes (Fuller et al. 2015; Montenegro-Montero et al. 2015). Curiously, a homolog of *frq* has been found in an arbuscular-mycorrhizal fungus in the Glomeromycota, *Rhizoglomus irregulare*, but how it originated in that species is not known (Lee et al. 2018). The version of the circadian rhythm controlled by *frq* was first documented in *N. crassa* in 1953, although the first example of circadian rhythm in fungi was found in *Pilobolus sphaerosporus* in 1951 (Schmidle 1951; Brandt 1953).

Control of the circadian rhythm in these species is due to an interplay between the *frq* protein and the WCC. The amount of *frq* present in the cell is dependent on the time of day and is controlled by the WCC. Late at night, the WCC induces the production of *frq* protein. As more *frq* protein is produced, it represses the activity of the WCC. Over time, the *frq* proteins are being phosphorylated, ubiquitinated, then broken down and discarded. As this happens, the WCC is released to induce production of the *frq* protein, forming the cyclical nature of the circadian rhythm (Dunlap and Loros 2006). The primary driver of the WCC cycle is WC1. While WC2 is present during the entire cycle, it is not differentially expressed at various times of day, unlike WC1 and *frq*, where the expression of the RNA and protein are linked to appropriate stages of the circadian rhythm (Dunlap and Loros 2006). VVD also has a minor effect on the circadian rhythm processes in *N. crassa*, being involved in the temperature compensation that maintains the circadian rhythm's regularity despite temperature variation; however, its involvement in circadian rhythm directly is primarily competing with frq for binding sites on the WCC, and helping to ensure the proper production of frq during the day (Heintzen et al. 2001; Hunt et al. 2007; Montenegro-Montero et al. 2015).

There may be other methods of controlling circadian rhythm that have not yet been discovered or analyzed. One potential system of controlling circadian rhythm was described in *Aspergillus flavus* and *A. nidulans* (Greene et al. 2003). In *A. flavus,* this rhythm is easily quantifiable by looking at the development of sclerotia, while the phenotype of *A. nidulans* dis not easily quantifiable, but studies of the *gpdA* gene indicate that it cycles in a circadian fashion. Neither *A. nidulans* or *A. flavus* have an ortholog of the frq gene found in *N. crassa* (Greene et al. 2003). As in *Aspergillus* species, the lack of a *frq* gene does not indicate that there is no circadian rhythm. The first fungus that was shown to have a circadian rhythm, *Pilobolus sphaerosporus*, has no *frq* gene, in common with many other related species (Schmidle 1951).

The circadian rhythm can control numerous functions in a fungal cell. The easiest ones to visually observe are pigmentation and reproduction. Pigmentation can be observed by rings or bands of colored hyphae, showing when the fungus grows differently during the night and day (Bluhm et al. 2010; Hubballi et al. 2010). Changes in reproduction can be seen as bands or rings of conidiation or sexual structures (Ingold and Cox 1955; Sargent et al. 1966; Austin (née Lowne) 1968; Greene et al. 2003). However, sometimes circadian rhythms cannot be observed as easily, as they cause no overt developmental changes, so the only way to test for their occurrence is by analyzing gene expression (Greene et al. 2003; Traeger and Nowrousian 2015). Because of this, it is likely that there are more fungi with functional circadian rhythms than have been reported to date.

1.7 Other methods of sensing and responding to light

While these proteins and methods of sensing and responding to light are very well conserved in many fungal lineages, they are not present universally. Most notably, most of these genes are missing from the yeasts *Saccharomyes cerevisiae* and *Schizosaccharomyes pombe,* with the exception of the photolyase proteins. Loss of the genes for light-sensing proteins appears to have occurred at least twice independently, as species in the *Scizosaccharomyces* genus, *S. japonicus,* and another species in the order *Saccharomycetales, Yarrowia lipolytica*, both still contain homologs for WC1 and WC2, indicating that WC1 and WC2 are ancestral (Niki 2014).

These fungal yeast lineages missing the WCC may have a different, indirect method of detecting light. In *Saccharomyces cerevisiae,* light damages cytochrome proteins, which then

produce reactive oxygen species. This activates the oxidative stress response, which also activates the pathways that allow tolerance to bright light (Robertson et al. 2013).

1.8 How does light stimulate a protective response in fungi?

There are multiple ways that a fungus can protect itself from the harmful effects of light and UV radiation. These include: producing photoprotective pigments, repairing damaged DNA, and detoxifying ROS.

1.8.1 Photoprotective compounds

Photoprotective pigments and other compounds that protect against UV radiation are very important for fungal survival because they absorb and disperse light energy before it can cause significant damage.

Melanin

Melanin is one of the ubiquitous pigments found in life across the kingdoms (True et al. 1999; Brenner and Hearing 2008; Gómez-Marín and Sánchez 2010; Vijayan et al. 2017; Eisenman et al. 2020; Pavan et al. 2020). There are multiple types of melanins found in fungi, depending on the pathways for their synthesis and the monomers of which they are composed (Figure 1.7). Little is known about the final structures of melanins, as melanins tend to form amorphous masses that are difficult to classify.

There are three major types of melanin found in fungi: allomelanin, eumelanin, and pyomelanin. Allomelanin, also known as dihydroxynaphthalene (DHN) melanin, is formed primarily but not exclusively by Ascomycete fungi (Figure 1.7) (Chumley 1990; Carzaniga et al. 2002; Butler et al. 2009; Eisenman and Casadevall 2012; Beltrán-García et al. 2014; Pal et al. 2014; Prados-Rosales et al. 2015; Islamovic et al. 2015; Fernandes et al. 2016; Derbyshire et al. 2018; Perez-Cuesta et al. 2020). This pigment is formed during secondary metabolism via a polyketide synthase pathway from acetyl-coA and malonyl-coA (Derbyshire 2018, Butler 2009, Moriwaki 2004). These melanins can present in different colors depending on the other proteins and compounds with which they are associated. *Aspergillus* species tend to produce green melanins,

while in most other species they are darker or even black (Carzaniga et al. 2002; Rahman et al. 2003; Choi and Goodwin 2011; Islamovic et al. 2015; Perez-Cuesta et al. 2020).

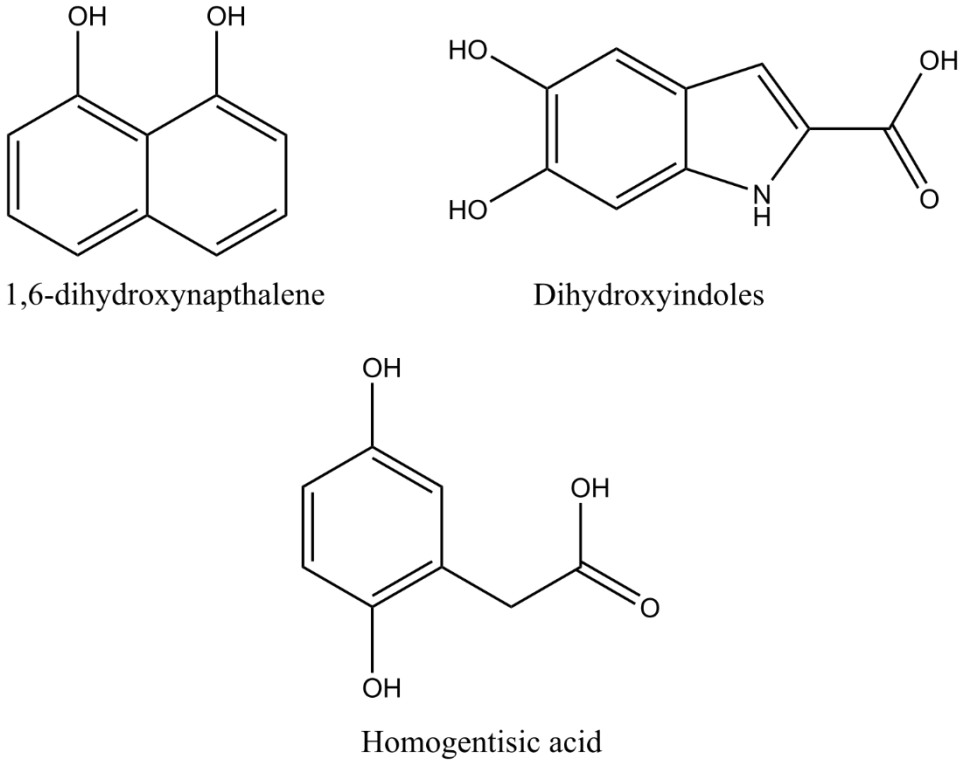


Figure 1.7 The precursors of the three melanins found in fungi.

The next type is eumelanin, also known as dihydroxyindole melanin (Figure 1.7). This melanin is found primarily but not only in basidiomycetes (Butler and Day 1998; Nagai et al. 2003; Chatterjee et al. 2012; Liu et al. 2014; Pal et al. 2014; Islamovic et al. 2015; Prados-Rosales et al. 2015). Fungi use laccases and phenoloxidases to produce eumelanin primarily from L-DOPA or tyrosine, but other substrates can be used (Garcia-Rivera et al. 2005; Eisenman et al. 2007; Nosanchuk et al. 2015). The formation and deposition of eumelanin has been well studied. *Cryptococcus neoformans* provides a good platform for analyzing the biosynthesis of eumelanin, as it is auxotrophic for the components and therefore requires them to be present in the growth medium.

The third, least-common melanin in fungi is pyomelanin, formed by a tyrosine degradation pathway (Figure 1.7) (Perez-Cuesta et al. 2020). L-tyrosine and L-phenylalanine are degraded to homogentisic acid (HGA), which spontaneously oxidizes to form pyomelanin (Perez-Cuesta et al.

2020). This form of melanin was discovered originally in bacteria, but now has been found in a variety of species across the fungal kingdom (Yabuuchi and Ohyama 1972; Coon et al. 1994; Goodwin and Sopher 1994; Carreira et al. 2001; Frases et al. 2007; van de Sande et al. 2007; Schmaler-Ripcke et al. 2009; Vasanthakumar et al. 2015; Almeida-Paes et al. 2016, 2018). Unlike the other two melanins, pyomelanins are soluble in water.

When eumelanin and allomelanin are formed, they are deposited in the fungal cell wall. They have multiple functions there, both to add strength and absorb harmful UV radiation, as well as to protect against other stresses. They are closely associated with chitin; when chitin production is disrupted, the ability to melanize the fungal cell wall also is lost (Bull 1970; Wang et al. 1999; Walton et al. 2005; Banks et al. 2005; Baker et al. 2007; Nosanchuk et al. 2015). The strength added to fungal cell walls by melanin can be a critical structural aspect of the cell wall, causing defects in cell development when it is missing (Tsai et al. 1998; Kawamura et al. 1999; Engh et al. 2007; Pihet et al. 2009; Yu et al. 2015).

Melanin is a very useful pigment, protecting the fungal tissue from more than just light. It protects against antifungal medicines, as well as having a role in resistance to fungicides (Van Duin et al. 2002; Ikeda et al. 2003; Paolo et al. 2006; Llorente et al. 2012; Baltazar et al. 2015; Lendenmann et al. 2015; Almeida-Paes et al. 2016; Fernandes et al. 2016; Toledo et al. 2017; Rossi et al. 2017). Highly melanized fungi occur in many extreme environments, ranging from Antarctica, to arid regions to high salt environments, to space stations, and to being able to survive in space itself (Grishkan et al. 2003, 2019; Novikova 2004; Selbmann et al. 2005; Gunde-Cimerman et al. 2006; Onofri et al. 2008, 2019; Ciccarone and Rambelli 2013; Pacelli et al. 2017). Highly melanized species are the most commonly found fungi in some of these areas, indicating that there is an evolutionary advantage to increased melanization in these areas. Melanin has also been shown to be critical in protecting fungi against other types of radiation than just solar. Interestingly, melanin has a use in transducing energy from ionizing radiation (Dadachova et al. 2007). The melanin is used to absorb gamma radiation and turn it into chemical energy that the fungus can use in a process similar to photosynthesis (Dadachova et al. 2007).

It should come as no surprise that light can induce the melanin production in various fungi (Singaravelan et al. 2008; Liu et al. 2010; Kihara et al. 2014; Wu et al. 2018; Du et al. 2020). In many species, this control is via the WCC (Sano et al. 2009; Kim et al. 2011c; Fuller et al. 2013; Pruß et al. 2014; Krobanan et al. 2019). In other species of fungi, melanin production also is

controlled by the velvet complex (Choi and Goodwin 2011; Wu et al. 2018). The control of melanin production via the velvet complex also may be driven by the WCC, as the velvet complex is intertwined with the WCC response, but that has not been confirmed.

Carotenoids

Another important class of pigments are the carotenoids. Carotenoids are fat-soluble, yellowish and reddish terpenoid-based pigments found in many realms of life. They occur in lipid globules and in plasma membranes inside cells, such as those found in the mitochondria or endoplasmic reticulum (Cederberg and Neujahr 1970; Keyhani et al. 1972; Riley and Bramley 1976; Mitzka-Schnabel and Rau 1980; Hsiao and Møller 1984; Arcangeli and Cannistraro 2000). These pigments are widely found in photosynthetic organisms like plants and cyanobacteria, as well as in many species of fungi (Cazzonelli and Pogson 2010; Kirilovsky and Kerfeld 2012; Avalos and Carmen Limón 2015). The best-known carotenoids in fungi are related to or derived from β-carotene, such as neurosporaxanthin and astaxanthin (Figure 1.8) (Avalos and Carmen Limón 2015).

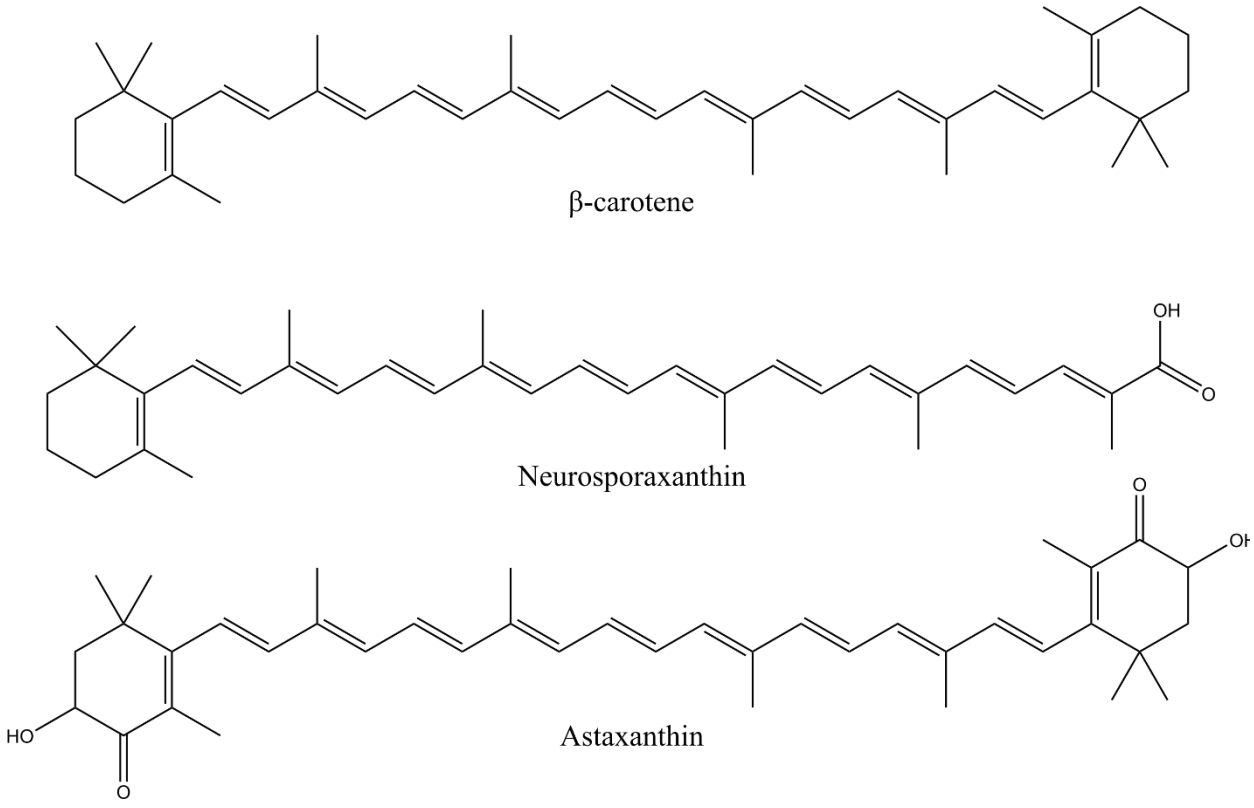


Figure 1.8 The chemical structure of carotenoids commonly found in fungi.

Carotenoids are very effective at quenching $^1O_2$ and the triplet state of photosensitizers, as well as acting as a photoprotective agent (Skibsted 2012). Because of this, they are commonly found in fungi, although they are not present in all species. Despite their common occurrence, they are missing from some model species, such as *S. cerevisiae* and *A. nidulans* (Avalos and Carmen Limón 2015).

With the high importance of carotenoids to photoprotection, it is not surprising that their production is highly regulated by light and the light-sensing proteins. The WCC controls the production of carotenoids in light-grown mycelia and conidia of multiple species, presumably to protect them from the energy in light (Harding and Turner 1981; Nelson et al. 1989; Toyota et al. 2002; Ruiz-Roldán et al. 2008; Kim et al. 2014a; Yang et al. 2016; Krobanan et al. 2019; Tang et al. 2020). One of the roles of VVD in *N. crassa* and *Cordyceps militaris* is the repression of carotenoid production, allowing them to produce enough carotenoids to protect the cells, but not enough to cause stress to the cells (Shrode et al. 2001; Zhang et al. 2020). It also has been found that the opsin nop1 in *N. crassa* has a role in regulating carotenoid production (Bieszke et al. 2007). The velvet complex also plays a role in regulating this process in *N. crassa* (Bayram et al. 2019). The high number of proteins involved in regulating carotenoid production indicates that this is an important function.

Mycosporines

While melanins and carotenoid pigments are widespread and well-studied in fungi, there is another photoprotective compound that is just as widespread, but much less well known, the mycosporine-like amino acids, or mycosporines. Mycosporines are not pigments like melanins and carotenoids; instead they are colorless compounds that absorb light at wavelengths between 268 and 362 nm (Wada et al. 2015). Mycosporines are found in bacteria, fungi and algae (Wada et al. 2015).

The six primary mycosporines in fungi are all based on aminocyclohexenone, with a cyclohexenone core and amino acids bound to a carbon (Figure 1.9). The fungal mycosporines all have a maximum absorbance at 310 nm (Leach 1965; Favre-Bonvin et al. 1976; Bouillant et al. 1981; Wada et al. 2015). There are six major types of mycosporines found in fungi, each with a different amino acid attached (Leach 1965; Trione et al. 1966; Tan 1975; Favre-Bonvin et al. 1976; Lunel et al. 1980; Bouillant et al. 1981; Fayret et al. 1981; Leite and Nicholson 1992, 1993; Oren

and Gunde-Cimerman 2007). Mycosporine-serinol was the first mycosporine identified, in the Basidiomycete *Stereum hirsutum*, but has been found in many other species since its initial discovery (Leach 1965; Favre-Bonvin et al. 1976). Mycosporine-glutamicol is also found in multiple species, including *Botrytis cinerea* and *Morchella esculenta* (Tan and Epton 1974; Bouillant et al. 1981; Fayret et al. 1981; Bernillon et al. 1984). Another two closely interlinked mycosporines are mycosporine-glutamicol-glucoside and mycosporine-glutaminol-glucoside. These two derivative mycosporines have been found in a wide variety of fungi across Dikarya (Trione et al. 1966; Bouillant et al. 1981; Volkmann et al. 2003; Kogej et al. 2006; Oren and Gunde-Cimerman 2007; Libkind et al. 2011). Mycosporine-serine is similar to mycosporine-serinol but has only been found in two species thus far (Lunel et al. 1980; Wada et al. 2015). Similarly, mycosporine-alanine has only been found so far in *Colletotrichum graminicola* (Leite and Nicholson 1992, 1993).

Mycosporines occur in both mycelia and in conidia and have been shown to function in more than just UV protection in fungal cells. They are involved in repressing sporulation in many species (Trione et al. 1966; Leite and Nicholson 1992, 1993). They also have been shown to function as solutes for salt tolerance in multiple fungi (Kogej et al. 2006; Oren and Gunde-Cimerman 2007).

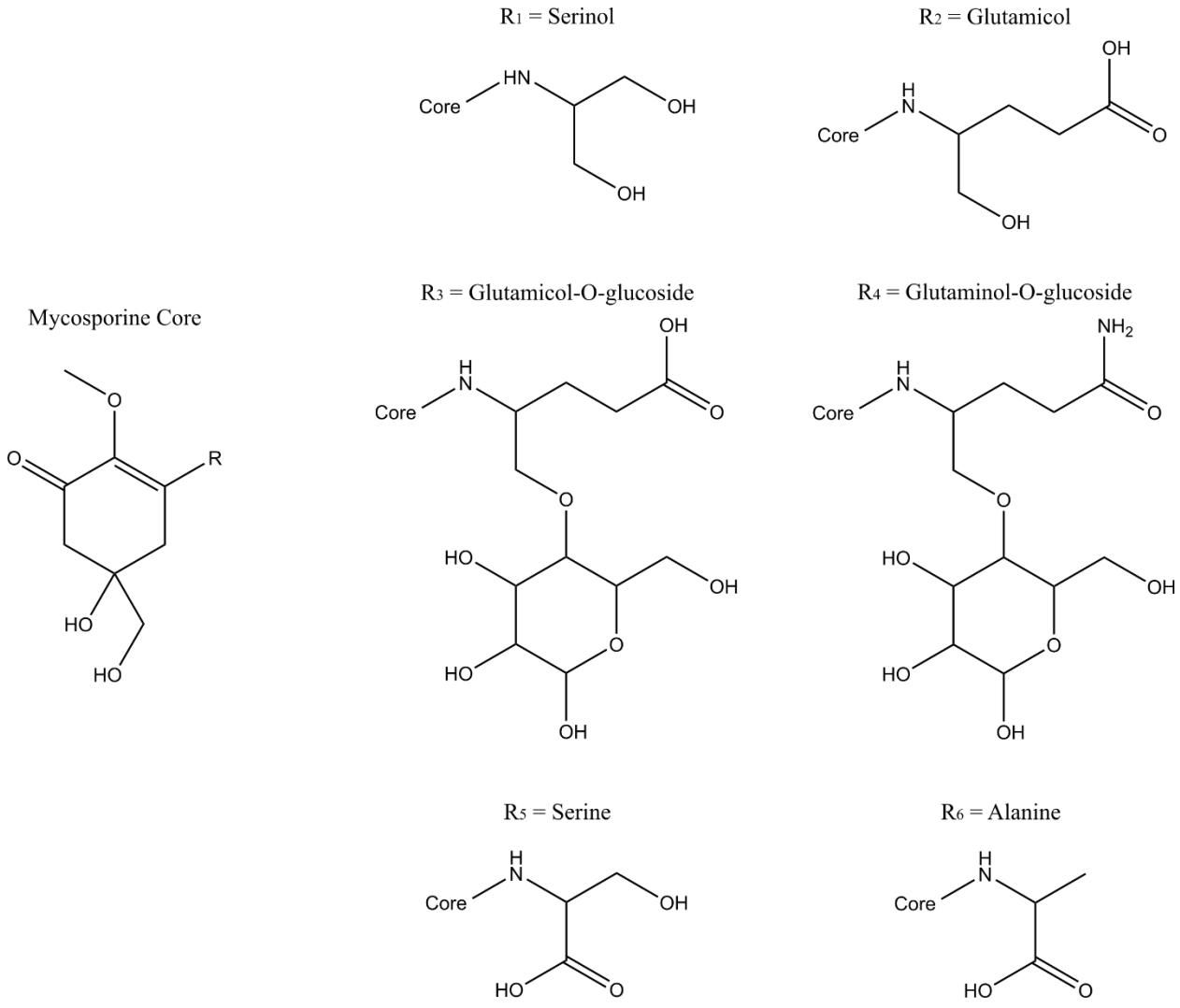


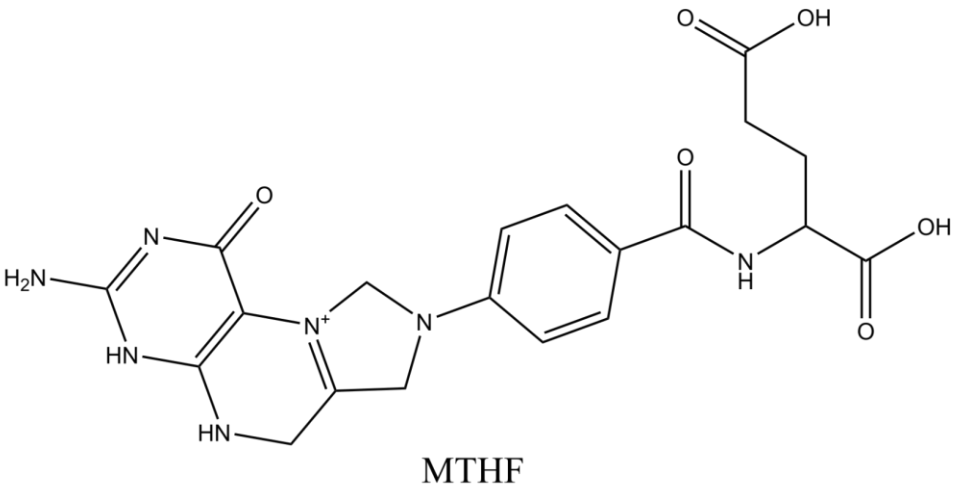
Figure 1.9 The structures of the mycosporine amino acid compounds. The cyclohexone core is shown to the left, and the six amino acid and amino acid derivative R groups are shown to the right.

1.8.2 DNA Repair

DNA damaged by UV radiation can be repaired by at least two methods. One is by repairing the nucleotides directly, and the second is by replacing the nucleotides. These are not the only mechanisms of repairing damaged DNA, but they are the ones that repair the damage done by UV light, so they will be the focus here.

Photolyase Repair

The first method of repairing DNA damage is by directly breaking the CPD and (6-4)PP bonds, returning the pyrimidines to their normal structures. This can be accomplished with specialized proteins known as photolyases. These proteins absorb blue light using a methenyltetrahydrofolate (MTHF) antenna, and the captured energy is transferred to the FAD cofactor and then used to repair the lesions (Figure 1.4, 1.10) (Eker et al. 1994; Rastogi et al. 2010). This process is known as photoreactivation.



MTHF

Figure 1.10 The structure of the MTHF antenna chromophore.

Many fungi contain only photolyases that can repair CPD lesions, but some fungi also contain a photolyase that can repair the (6-4)PP lesions (Bayram et al. 2008a; Bluhm and Dunkle 2008; Inoue 2011; Fang and St. Leger 2012; Wang et al. 2019a).

Photolyases do the most good when they are produced at the times and locations where they will be needed. As such, photoreactivation takes place in the light. It is well known that when an organism is irradiated by UV light, and then placed in the dark, it has much lower survivability than when placed under light. This provides a laboratory test for photoreactivation (Kelner 1949; Goodman 1958; Townsend 1961).

Of course, a key aspect of this process is that the photolyase requires energy from blue light to repair the lesions found in UV-damaged DNA, thus many fungi produce photolyases when blue light is present (Alejandre-Durán et al. 2003; Kihara et al. 2007; McCorison and Goodwin 2020). To ensure this, many species utilize the WCC to control expression of photolyases

(Berrocal-Tito et al. 2007; Ruiz-Roldán et al. 2008; Fuller et al. 2013; Ohm et al. 2013; Cervantes-Badillo et al. 2013; García-Esquivel et al. 2016; Brych et al. 2016). The blue-light sensing protein complex is an ideal candidate to stimulate photolyase-induced DNA repair, as in nature blue light is often found concurrently with damaging UV radiation.

Nucleotide Excision Repair

The other method of repairing UV-damaged DNA is the nucleotide excision repair (NER). In this process, proteins recognize damaged nucleotides, either resulting from UV or other sorts of DNA damage, and a section of DNA surrounding the faulty base is excised and replaced. There are multiple variations on this process, using most of the same protein complexes to do the bulk of the work. This method is much more complicated and slower than using photolyases, but also is more flexible, able to repair damage that cannot be fixed by photolyases.

The primary version of NER, found in organisms ranging from *S. cerevisiae* to *Homo sapiens*, utilizes over 20 proteins assembled into seven complexes (Boiteux and Jinks-Robertson 2013). The first step in the repair process occurs when a protein complex recognizes the lesion and opens the DNA into single strands. After that, the DNA around the lesion is widened using a pre-incision protein complex that holds the opening stable. Two endonucleases bind to the pre-incision complex to cut and remove the single-stranded DNA surrounding the lesion. The gap is filled in by polymerases and finally reattached via a DNA ligase.

Most of the versions of NER differ in how the lesion is detected. One method of detection, found in *S. cerevisiae*, is based on RNA polymerase II (RNAPII). When RNAPII encounters a lesion on an active strand of DNA, transcription is stalled. A protein, known as Rad26 in *S. cerevisiae*, binds to the polymerase and causes it to reverse and back away so the rest of the NER pathway can remove the lesion, or it can bypass the lesion, leading to an mRNA that contains an error (Boiteux and Jinks-Robertson 2013).

In *Schizosaccharomyces pombe*, *N. crassa*, and *A. nidulans*, there is a repair pathway specific for UV damage known as alternative excision repair (AER) (Yajima et al. 1995; Yonemasu et al. 1997; Goldman and Kafer 2004). This pathway uses a UV damage-specific endonuclease instead of those used in standard NER (Yonemasu et al. 1997; Ishii et al. 1998). The use of AER leads to faster repair of the DNA lesions. It has been shown in two studies that these UV-specific NER pathways are activated by exposure to light. In a study in *Cryptococcus*

neoformans, it was determined that the AER pathway is initiated by the WCC (Verma and Idnurm 2013).

1.8.3 Detoxification of Reactive Oxygen Species

One strategy that fungi use to mitigate the effects of light-generated ROS is to detoxify the damaging compounds before they can cause too much damage. This method has the additional function of detoxifying ROS generated by other means than by light exposure, such as those caused by an oxidative burst as plants defend themselves, or by general aerobic respiration (Lendenmann et al. 2015). There are two primary methods for detoxifying ROS: using antioxidants to reduce the ROS; and producing enzymes that reduce the ROS to less reactive states.

Antioxidants can bind to and neutralize ROS so they cannot cause damage (Skibsted 2012). All the photoprotective compounds mentioned above also can act as effective antioxidants. Other antioxidants that can be found in fungi are the flavonoids, glutathione and other thiols, ascorbate, and phenolic compounds (Belozerskaya and Gessler 2007; Garrido-Arandia et al. 2016). Carotenoids especially are efficient scavengers of singlet molecular oxygen and peroxyl radicals (Skibsted 2012). These pigments and antioxidants also function to dissipate energy from photosensitizers and return compounds to unexcited states.

A few different enzymes can be used to detoxify ROS. A major class of ROS-detoxyfying enzymes are the superoxide dismutases. These enzymes react with superoxide (O^{-}_{2}) and the hydroxyl radical ($^{\bullet}OH$) to form oxygen and hydrogen peroxide. While hydrogen peroxide is also considered an ROS, there are multiple enzymes that can detoxify the hydrogen peroxide formed by superoxide dismutases or from other sources, while the superoxide dismutase enzymes are the only ones that can detoxify superoxide. These other enzymes, the catalases and the peroxidases, split hydrogen peroxide into water and/or oxygen, depending on the class (Mayer et al. 2001).

Light can play a role in oxidative stress tolerance, and it has been shown to directly affect the transcription of various genes used to detoxify ROS (Carreras-Villaseñor et al. 2012; Fuller et al. 2013; Wu et al. 2014). In some fungal species, this is done via the various photosensing proteins, such as the WCC or phytochrome (Canessa et al. 2013; Fuller et al. 2013; Qiu et al. 2014; Igbalajobi et al. 2019). The velvet complex also can play a role in regulating the transcription of these ROS-detoxifying enzymes (Yang et al. 2013a; Gao et al. 2017; Wu et al. 2018; Zhang et al. 2018; Wang et al. 2019c). Curiously, in some speciess such as those in the genus *Trichoderma*,

oxidative stress can tie into the light-response pathways, and be required for some of the photoresponses such as conidiation (Friedl et al. 2008; Schmoll 2018).

1.9 How do phytopathogenic fungi use the light beyond protection?

Fungi use light as a cue to initiate many different processes. Pigmentation and responses to ROS are only a small part of the total when it comes to fungal responses to light.

1.9.1 Light cues how to grow and reproduce

Fungi often use light as a cue to regulate general growth rate and patterns. These patterns can vary widely from species to species. For instance, while white light encourages greater growth *in vitro* in *Aspergillus* species, it reduces the growth rate in species of *Penicillium*, another closely related genus in the Eurotiales, and in *Fusarium* and *Alternaria* species, light has no effect on growth rates at all (Schmidt-Heydt et al. 2011). In other species, different wavelengths of light can affect growth rates differently (Yu et al. 2013).

In many species, light can induce the production of conidiophores and asexual structures and repress the formation of sexual structures (Lauter and Russo 1991; Calvo et al. 1999; Blumenstein et al. 2005; Schmoll et al. 2005; Bayram et al. 2010a; Olmedo et al. 2010). In other species, light does the opposite, repressing the production of asexual structures while inducing the formation of sexual structures (Lukens 1963; Flaherty and Dunkle 2005; Lee et al. 2006; Bluhm et al. 2010; Kim et al. 2011a).

Many of these responses are directly controlled by the various light-sensing proteins in fungi. The WCC and VVD have both been linked to controlling reproduction, both sexual and asexual, in many species of fungi in response to blue light. In many of these species, conidiation is repressed by these proteins in the light (Lee et al. 2006; Kim et al. 2011a; Pruß et al. 2014; Krobanan et al. 2019). In some species the opposite is true, and these proteins instead induce conidiation (Olmedo et al. 2010; Canessa et al. 2013; Yang et al. 2016; Tong et al. 2018; Zhang et al. 2020). The effects of light on sexual reproduction are just as varied, with the WCC required for the production of some sexual structures, and in other cases repressing their development (Purschwitz et al. 2008; Seibel et al. 2012; Canessa et al. 2013; Kim et al. 2014a; Yang et al. 2016; Bazafkan et al. 2017; Schmoll 2018; Sánchez-Arreguin et al. 2019; Krobanan et al. 2019; Tang et

al. 2020). Similarly, the phytochrome protein has been shown to affect sexual and asexual reproduction. It has been linked to repression of sexual development in *Aspergillus nidulans* but is required for sexual development in *Ustilago maydis* (Purschwitz et al. 2008; Sánchez-Arreguin et al. 2019). It also can induce conidia formation in *Alternaria alternata* and *Aspergillus nidulans* (Blumenstein et al. 2005; Igbalajobi et al. 2019). The velvet complex also is critical in many species for controlling light-regulated reproduction, either by inducing conidiation or repressing it, depending on the species of fungus, and by controlling sexual reproduction as well (Kim et al. 2002; Calvo et al. 2004; Duran et al. 2007; Ni and Yu 2007; Bayram et al. 2008c; Duran et al. 2009; Merhej et al. 2012; Karakkat et al. 2013; Yang et al. 2013a; Kim et al. 2014b; Schumacher et al. 2014; Bazafkan et al. 2015; Rahnama et al. 2018; Wu et al. 2018; Zhang et al. 2018; Tiley et al. 2019; Wang et al. 2019c).

Germination rates of spores can also be controlled by light. The spores, sexual and asexual, may not germinate when light is present, presumably due to the additional stresses caused to an exposed germ tube before it can invade the plant host (Willocquet et al. 1996; Sun and Yang 2000; Leonard and Szabo 2005; Fuller et al. 2013; Yu et al. 2013; Röhrig et al. 2013).

1.9.2 Light can cue when to produce secondary metabolites

Pigmentation is just one aspect of secondary metabolism that phytopathogens regulate based on light and light-initiated cues. Many other secondary metabolites are produced by fungi depending on light conditions. These other secondary metabolites include various mycotoxins and antibiotics. Some of these are toxins that affect the plant host to cause disease, some are produced by the plant but harm humans and livestock, and yet others function as antibiotics to prevent other organisms from colonizing the same plant (Aziz and Moussa 1997; Daub et al. 2005; Ostry 2008; Abeysekara et al. 2009; Schmidt-Heydt et al. 2011; Fanelli et al. 2012a; Mulrooney et al. 2012; Kim et al. 2014a). Many mycotoxins can cause both pre- and post-harvest problems in agricultural production, while others cause problems primarily before harvest (Yuen and Schoneweis 2007; Klich 2007; Wang et al. 2016b; Caceres et al. 2020).

The production of many mycotoxins is controlled by light (Soderhall et al. 1978; Bayram et al. 2008b; Schmidt-Heydt et al. 2011; Kim et al. 2014a). The most common wavelength of light that controls this is blue light, which can repress or induce the production of these mycotoxins,

depending on the species and toxin in question (Purschwitz et al. 2008; Bayram et al. 2010b; Schmidt-Heydt et al. 2011; Kim et al. 2014a; Suzuki 2018).

Since light affects production of these secondary metabolites, the light-sensing proteins also are involved in the production of these toxins. The WCC and phytochrome proteins can both play a major role in regulating the production of many of these toxins. In some cases, they work to induce the production of the mycotoxin, and in others they work to repress toxin formation (Estrada and Avalos 2008; Purschwitz et al. 2008; Kim et al. 2014a; Pruß et al. 2014; Igbalajobi et al. 2019). The velvet complex has been shown to be a critical factor in controlling mycotoxin production in several species (Kato et al. 2003; Duran et al. 2007, 2009; Spröte and Brakhage 2007; Bayram et al. 2008b; Merhej et al. 2012; Wang et al. 2019b).

1.10 How does this tie into pathogenicity?

The light-related processes mentioned so far relate to pathogenicity, but only indirectly. Cuing sporulation at the right time does not affect the ability of a pathogen to infect, but because the spores are produced when they will have a higher survival, it leads to higher effective inoculum levels, which does affect pathogenicity. This is not a direct effect, where light affects ability of the pathogen to penetrate and grow within its host; however, the indirect effect on inoculum levels can affect the overall pathogenicity of the species.

1.10.1 Plant Defense Systems

The ability of plants to defend themselves from phytopathogens is well-known and well-studied. Plants can regulate defense mechanisms using light. Plants have three primary photoreceptors that they use to induce expression of their pathogen defense mechanisms. These are the phytochromes and cryptochromes, which are related to the phytochrome and cryptochrome proteins in fungi as described above, and a different photoreceptor known as UVR8, which can detect UV-B light (280 – 315 nm) and has no homolog in fungi (Sharrock and Quail 1989; Ballaré 1999; Brudler et al. 2003; Sellaro et al. 2010; Rizzini et al. 2011; Keller et al. 2011; Liu et al. 2016).

Plants have two primary defense-signaling pathways, one using jasmonate, and the other using salicylic acid. The jasmonate pathway leads to a cascade of gene expression changes and the

induction of plant defense mechanisms (Penninckx et al. 1998; Shan et al. 2007; Ballaré 2011; Kazan and Manners 2012). The salicylic acid pathway is best known for leading to systemic acquired resistance (SAR), a broad-spectrum disease resistant response in plants (Senaratna et al. 2000; Uppalapati et al. 2007; Wu et al. 2012; Fu and Dong 2013). The jasmonate and salicylic acid pathways interact in a mutually antagonistic way to appropriately coordinate a defense against pathogens and herbivores (Spoel et al. 2007; Ballaré 2011; Pieterse et al. 2012).

Sometimes, these defense responses are dependent upon light. The primary way that light affects these two pathways is the phytochrome and the red:far red light differences. Far red light is known to repress the jasmonate pathway, while stimulating the salicylic acid pathway, and SAR and the hypersensitive response are much more robust in the light (Genoud et al. 2002; Griebel and Zeier 2008; Moreno et al. 2009; Kazan and Manners 2011; Cerrudo et al. 2012; De Wit et al. 2013; Ballaré 2014). Plants that have mutations in the phytochrome gene are more susceptible to biotrophic and hemi-biotrophic pathogens (Griebel and Zeier 2008; Wang et al. 2010; Xie et al. 2011; Cerrudo et al. 2012). There has been some study as to whether UVR8 and the cryptochrome gene play a role in pathogen resistance, but the results have been inconclusive (Yalpani et al. 1994; Izaguirre et al. 2003; Caputo et al. 2006; Griebel and Zeier 2008; Wu and Yang 2010; Demkura et al. 2010; Jeong et al. 2010; Cerrudo et al. 2012; Morales et al. 2013). Despite this, circadian rhythm can affect both pathways, and therefore it is very important for plant immune responses (Griebel and Zeier 2008; Goodspeed et al. 2013b, a; Zhang et al. 2013; Karapetyan and Dong 2018; Yamaura et al. 2020).

1.10.2 Light and Pathogenicity

Light can affect the phytopathogen as well. Sometimes there is a strong correlation, and the intensity and wavelength of light can affect the virulence of the pathogen directly (Kim et al. 2011c; Yu et al. 2013). There are also times when the circadian rhythm can directly affect the virulence, such as in *Botrytis cinerea* (Hevia et al. 2016).

Oftentimes this is discovered as a consequence of manipulating the various light-sensing genes within the fungus. WC1 is a common target for knockouts, and thus there have been multiple species found where it has an effect on pathogenicity and virulence. In *Botrytis cinerea*, WC1 knockouts have reduced virulence in full light, but can still infect at normal virulence when plants are left in full darkness (Canessa et al. 2013). The reason for this is not entirely clear, however,

WC1 also affects the ability of *B. cinerea* to cope with oxidative stress, as well as its general growth patterns (Canessa et al. 2013). In the rice pathogen *Magnaporthe oryzae*, light exposure immediately after inoculation represses disease development, but in the WC1 knockout mutant, that repression is partially undone (Kim et al. 2011c). In *Cercospora zeae-maydis*, WC1 mutants are completely avirulent (Kim et al. 2011a). Microscopic examinations of inoculated corn plants show that germ tubes are unable to sense and invade stomata, and instead grow right over them without penetration (Kim et al. 2011a). It is well known that *C. zeae-maydis* is non-thigmatrophic when it comes to sensing stomata, but the precise mechanism is unknown (Rathaiah 1977; Beckman and Payne 1982).

The velvet complex has been found to affect virulence and pathogenicity in multiple species of fungi throughout the fungal kingdom. In *M. oryzae*, two velvet genes affect the development of appressoria, preventing full virulence of the pathogen on the host when mutated (Kim et al. 2014b). In *Ustilago maydis*, the two velvet genes affect how well the fungus proliferates through the host, preventing full virulence when both are knocked out (Karakkat et al. 2013). In *B. cinerea* and *A. flavus*, when the homolog to *veA* was disrupted, the ability to colonize plant tissue was impaired. These phytopathogens were able to infiltrate the host, however, they could not further colonize it (Duran et al. 2009; Schumacher et al. 2015). In *F. graminearum* and *Valsa mali*, it is hypothesized that the velvet complex affects production of virulence factors, and toxins or pectinases, respectively, which enhance the virulence of these phytopathogens, and when members of the velvet complex are disrupted, so is production of those virulence factors with a concomitant decrease in pathogenicity (Merhej et al. 2012; Wu et al. 2018).

Multiple papers have proposed that opsins are involved in pathogenicity and virulence because the phyllosphere is primarily exposed to green light, and opsin proteins are green light driven or green light sensors (García-Martínez et al. 2015; Lyu et al. 2016; Adam et al. 2018; Panzer et al. 2019). Opsins are more consistently present in plant pathogens, which supports this hypothesis (García-Martínez et al. 2015; Adam et al. 2018). While fungal opsins are not a widely studied class of proteins, some analyses have shown that they play a role in pathogenicity and virulence. In the generalist pathogen *Sclerotinia sclerotinium*, knockouts of the LR-like opsin *sop1* show lower virulence (Lyu et al. 2016). However, in *Fusarium fujikuroi*, knockouts of the carO opsin showed greater virulence, but not the NR-like OpsA (Adam et al. 2018). One hypothesis explaining these results in *F. fujikuroi* is that carO opsin represses germination. When it is knocked

out, the spores germinate faster, thus causing symptoms faster in a susceptible host (Adam et al. 2018).

Melanin as a Virulence Factor

Melanin plays additional roles in phytopathogens in addition to serving as a photoprotective compound. Melanin can be a critical component of the ability in many phytopathogens to infect plants by acting in other ways.

The primary role that melanins play in pathogenicity is as a structural component in the cell wall, specifically in the appressoria of phytopathogens (Kubo et al. 1982; Woloshuk et al. 1983; Chen et al. 2004; Gachomo et al. 2010; Toledo et al. 2017). The turgor pressure in appressoria is high, and without the structural aid of melanin, the appressoria are unable to form cell walls strong enough to invade the plant, hampering the ability of the fungus to infect. This is not true for all appressoria-forming phytopathogens, but it is true for many (Kawamura et al. 1999; Saitoh et al. 2010; Chang et al. 2014). This also means that melanin-synthesis pathways are a target for fungicides, such as carpropamid and tricyclazole, which interfere with allomelanin biosynthesis (Woloshuk et al. 1983; Tsuji et al. 1997).

In at least one phytopathogen, a type of melanin plays a more direct role in pathogenicity. Allomelanin may be a virulence factor in the banana pathogen *Pseudocercospora fijiensis*. (Beltrán-García et al. 2014). Allomelanin from *P. fijiensis* forms singlet molecular oxygen when irradiated with light, indicating a potential to function as a light-activated toxin. Additionally, byproducts from melanin shunt pathways in *P. fijiensis* have been shown to be toxic to plants (Stierle et al. 1991; Hoss et al. 2000). Treatment of banana plants infected with *P. fijiensis* with tricyclazole worsens disease symptoms and leads to a greater buildup of these shunt pathway byproducts than occurs in control plants, indicating that these shunt byproducts play a role in phytotoxicity (Hoss et al. 2000).

Melanin can have another, indirect effect on virulence. Melanin can play a major role in resistance to fungicides and other antifungal agents by binding them and reducing their ability to damage the pathogen (Llorente et al. 2012; Lendenmann et al. 2015). This phenomenon has been studied more in relation to animal pathogens and antifungal drugs, but there has been some limited research in the role of melanin as a protective agent in phytopathogens as well, since if the fungus can resist fungicides used to control it, the disease pressure will be greater.

Toxins

Light is well known to be a major contributor in controlling toxin production in various species of fungi. This was discussed earlier regarding mycotoxins found in plants that can harm humans and livestock, but light can also affect the production of toxins that harm the plants and lead towards higher virulence and damage.

One major class of toxins produced by fungi is the photosensitizers. These mycotoxins form ROS when they are exposed to UV-A and visible light. They are commonly used by fungal pathogens to cause massive amounts of damage to their plant hosts (Foote 1976).

Many photosensitizers are perylenequinones. These are synthesized by phytopathogenic fungi in the Ascomycetes, especially those in the class Dothideomycetes (Daub et al. 2005). One of the best known perylenequinones is cercosporin, produced by fungi in the genus *Cercospora* in the Dothideomycetes (Figure 1.11) (Daub et al. 2005; Mulrooney et al. 2012). Cercospoin is highly toxic to any form of life, save other species that produce perylenequinone toxins (Daub 1987). Due to this, *Cercospora* species also have a high resistance to photosensitizers of any sort (Daub et al. 1992; Jenns et al. 1995). One important factor to note is that cercosporin is necessary for full virulence of the *Cercospora* species in which it is produced (Daub et al. 2005). Strains that have lost the ability to produce cercosporin do not form as many lesions as the wild type strain, and the lesions produced are much smaller (Daub et al. 2005). The production of cercosporin is well known to be stimulated by the presence of light, likely due to its photoreactive properties, and has been found to be controlled by the WCC (Kim et al. 2011a).

While cercosporin is one of the best known perylenequinone toxins, several other compounds in this same group are used by phytopathogens. *Alternaria* species produce the perylenequinone-based altertoxins (Figure 1.11) (Stack et al. 1986; Scott 2001; Ostry 2008). Like cercosporin, the altertoxins cause major cellular damage (Hartman et al. 1989; Scott 2001; Ostry 2008); the production of altertoxin I is also highly controlled by light and the homologs of the WCC in *Alternaria alternata* (Pruß et al. 2014). *Shiraia bambusicola* produces the perylenequinone toxin hypocrellin A at higher rates when cultured under constant light, specifically red light (Figure 1.11) (Sun et al. 2018; Ma et al. 2019). There are other perylenequinones produced in fungal species; however, there have not been any studies that link their production to light (Overeem et al. 1967; Robeson et al. 1984; Stierle et al. 1989; Davis and Stack 1991; Robeson and Jalal 1992; Daub et al. 2005; Stergiopoulos et al. 2013). It is entirely

possible that the production of these perylenequinones is controlled by light and the WCC, but this hypothesis remains to be tested.

A correlation between light intensity and disease severity has been found in other Dothideomycetes fungal species (Echandi-Zürcher 1959; Calpouzos 1966; Rotem et al. 1988; Daub and Ehrenshaft 2000). In the past, one recommended method of control of yellow sigatoka disease, caused by *Mycosphaerella musicola*, was to shade banana plants, as reducing the amount of light reduced the disease symptoms (Thorold 1940; Calpouzos and Corke 1963; Calpouzos 1966). A similar relationship to light has been found in other *Cercospora* species (Echandi-Zürcher 1959; Daub and Ehrenshaft 2000). These tendencies, when paired with the knowledge that photoactivated perylenequinone-based toxins are common in Dothideomycetes, suggests that these changes in disease virulence may be a sign of the presence of a perylenequinone-based toxin in these other pathogens.

Of course, while there are several known photoactivated toxins, there are others whose expression is simply controlled by light. *Alternaria* species are an excellent source of toxins, as they make a wide variety of different compounds depending on the species and strain (Scott 2001; Ostry 2008). Production of some of these other toxins, such as alternariol and alternariol monomethyl ether, also is controlled by light (Figure 1.11) (Soderhall et al. 1978; Haggblom and Unestam 1979; Igbalajobi et al. 2019). However, the production of these two toxins is inhibited by light, as opposed to the altertoxins, production of which is stimulated by light (Soderhall et al. 1978; Haggblom and Unestam 1979; Pruß et al. 2014; Igbalajobi et al. 2019). Similarly, in the Leotiomycetes fungus *Botrytis cinerea*, the toxins botcinic acid and botryniol are used to harm plant tissues, and the productions of these are both controlled by the Velvet complex and the light-responsive transcription factor 1 (Figure 1.11) (Schumacher et al. 2014, 2015). All these mycotoxins are important for pathogenicity and virulence of these pathogens, and knowing what triggers are used to induce and repress their production can be a critical component of disease-control strategies (Dalmais et al. 2011; Graf et al. 2012; Wojciechowska et al. 2014).

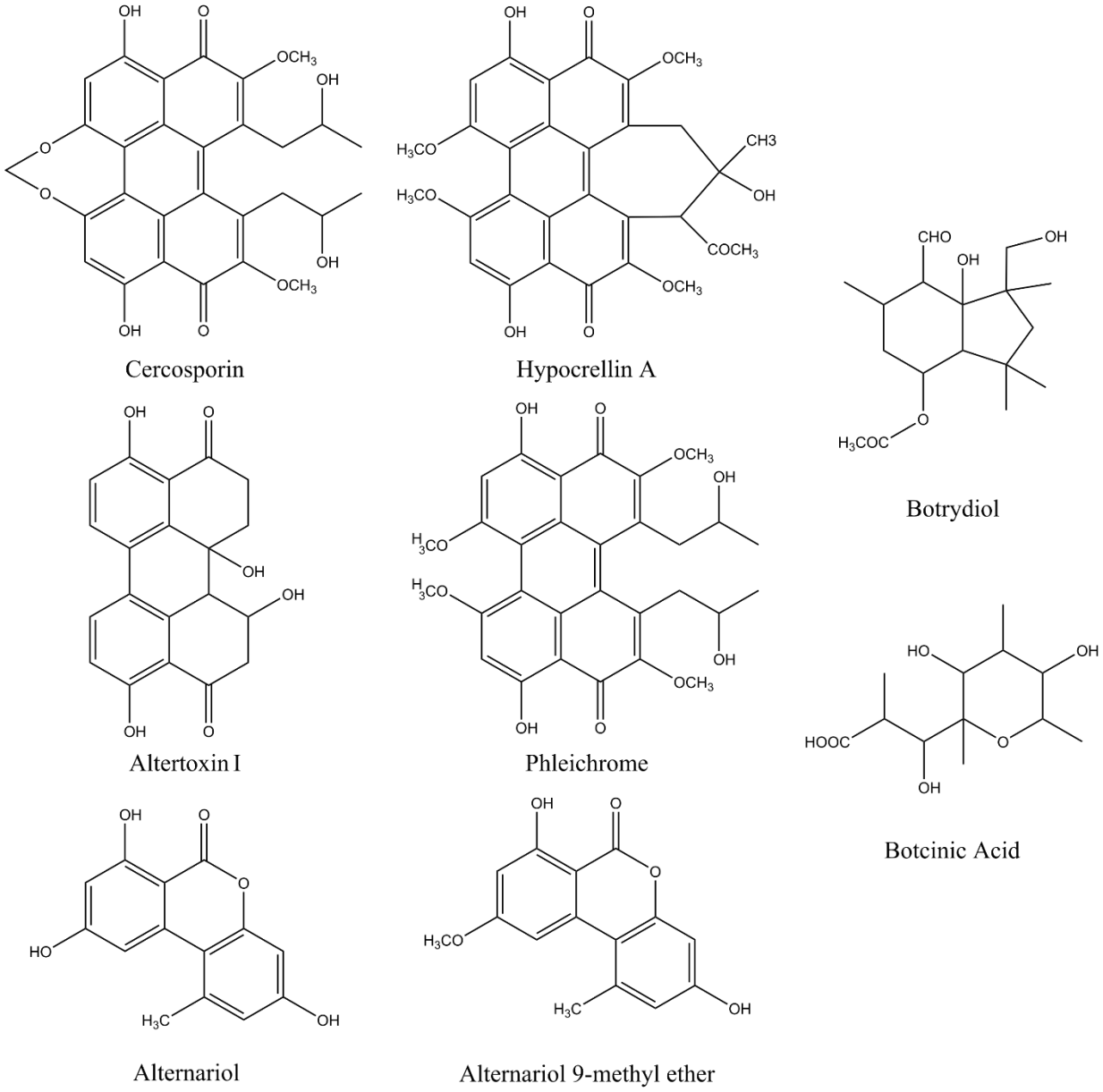


Figure 1.11 Chemical structures of a selection of toxins found in fungal phytopathogens.

1.10.3 Other Considerations:

Light-sensing proteins and those regulated by light can play other roles in pathogenicity and virulence. These roles may be smaller in scope, limited to individual species, or not directly related to pathogenicity at all.

Regarding the latter point, some of these light-related phenomena affect the tolerance of these phytopathogens to fungicides. As mentioned previously, melanins are well known to protect fungi from antifungal drugs in animals, however they also have been shown to play a role in protecting phytopathogenic fungi from fungicides in some species (Frederick et al. 1999; Van Duin et al. 2002; van de Sande et al. 2007; Lendenmann et al. 2015; Almeida-Paes et al. 2016; Fernandes et al. 2016). In one species, *Sclerotinia sclerotiorum*, a nop1-like opsin is involved in fungicide tolerance, and disruption of the gene causes a susceptibility to the tested fungicides (Lyu et al. 2016; Wang et al. 2017b).

In *Fusarium fujikuroi*, a CarO opsin modifies the virulence of the fungus (García-Martínez et al. 2015; Adam et al. 2018). This opsin protein represses and thereby slows the germination of spores (García-Martínez et al. 2015). Under laboratory conditions, the CarO mutant strains were much more aggressive, with the rice hosts showing much more severe symptoms than when infected with the wild type (Adam et al. 2018). This was due to a greater fungal biomass, indicating a potential link between the two functions of the CarO opsin in *F. fujikuroi*, where faster germination leads to more severe symptoms. While this is true under laboratory conditions, which are often ideal for fungal infection, it may not be true under field conditions. While faster germinating spores can outcompete other fungi, it also may lead to spore germination before conditions for infection are favorable, which may be maladaptive in nature. The green-light-sensing CarO opsin may be involved because of the reflection of green light from the surface of the host plant.

A final consideration is that light can be used in the control of various pathogens. Methods have been developed for using UV light to kill phytopathogenic spores before they are able to germinate and successfully infect a plant (Willocquet et al. 1996; Raviv and Antignus 2004; Suthaparan et al. 2012; Kobayashi et al. 2013). Other research has been done on using visible light to reduce the growth of pathogens, or to delay their infection (Islam et al. 2002; Romero Bernal et al. 2019; Radetsky et al. 2020). The use of light as a potential control mechanism may be useful, however it is not foolproof. Fungi possess several means for protecting themselves from light, but when used with other methods of control, light manipulation may add another tool to the arsenal available to fight plant diseases.

1.11 Conclusions

Understanding the role played by light in the pathogenicity and virulence of phytopathogens is a very important step in understanding how to manage plant diseases in the future. The scientific community is becoming more interested in the mechanisms used by phytopathogens to respond to light, yet the more that is learned, the more questions are unearthed. The highly variable responses to light, even within a single genus or species, indicate that current hypotheses generated regarding how a species will respond to light may not be correct, even when they may be supported by evidence from closely related species.

1.12 *Zymoseptoria tritici*

Zymoseptoria tritici (Desm.) Quaedvlieg & Crous (syn. *Mycosphaerella graminicola, Septoria tritici*) is a fungal pathogen of wheat (*Triticum aestivum* and *T. turgidum* ssp. *durum*) that causes the disease Septoria tritici blotch (STB). *Z. tritici* is in the phylum Ascomycota, class Dothideomycetes, order Capnodiales, family Mycosphaerellaceae. Many well-known phytopathogens are also in the Dothideomycetes, such as *Alternaria alternata,* which causes leaf spot and stem canker diseases on a wide range of host plants, and *Cercospora zeae-maydis* and *C. zeina*, which cause grey leaf spot on corn (*Zea mays*).

STB is a major disease of wheat, particularly in Europe and the UK; however, it can be found anywhere wheat is grown. This disease can be devastating to yields if left unchecked. STB causes massive amounts of yield loss if left uncontrolled during an epidemic year; losses can reach up to 50%, and even in years where it is controlled, small losses of 5-10% can be seen (Eyal 1973; Eyal et al. 1987; Fones and Gurr 2015). Up to 70% of fungicides used in the field in the EU are used to prevent STB (Fones and Gurr 2015; Torriani et al. 2015). Control of *Z. tritici* is primarily done via genetic resistance and fungicides (cite?).

The genome of *Z. tritici* has been fully sequenced and assembled into a complete genome (Goodwin et al. 2011). The genome of the sequenced strain, the Dutch isolate IPO323, is 39.7 Mb long, divided into 21 chromosomes (Kema and Van Silfhout 1997; Goodwin et al. 2011). Of those 21 chromosomes, 8 are dispensable and unnecessary for normal growth and pathogenicity (Goodwin et al. 2011; Dhillon et al. 2014). These 8 dispensable chromosomes are highly divergent

from the 13 core chromosomes, with lower GC content, higher amounts of repetitive DNA, and a lower gene density (Goodwin et al. 2011; Dhillon et al. 2014).

1.13 Life and Infection Cycle

The lifecycle of *Z. tritici* begins with an initial inoculum of primarily airborne ascospores originating from pseudothecia present on wheat debris from the previous growing season. These land on seedling leaves and germinate, and the hyphal tubes invade via the stomata. This process requires high humidity to ensure the germ tubes do not dry out. Over the next 12-14 days, depending on host cultivar and pathogen strain, hyphae grow through the apoplast between the mesophyll cells, causing few to no symptoms and gaining little biomass. This is known as the latent phase. *Z. tritici* switches to necrotrophic growth, killing the plant tissue it has grown through, and forming yellow and chlorotic, then brown, necrotic lesions embedded with dark pycnidia. These pycnidia are formed in the stomata of the wheat leaves. The pycnidia exude conidia in thick cirrhi and those conidia are spread via rain splash to the upper leaves of the same plant or to nearby plants. After more time, and provided a suitable mate, pseudothecia also form within the stomata of the host plant. The ascospores produced are disseminated by wind, allowing the infection to spread further (Figure 1.12) (Kema et al. 1996; Ponomarenko et al. 2011). *Z. tritici* is a hemibiotrophic fungus, due to the long latent phase followed by a necrotrophic phase. However, this has also been debated recently, with some calling it to be reclassified at a latent necrotroph (Sánchez-Vallet et al. 2015).

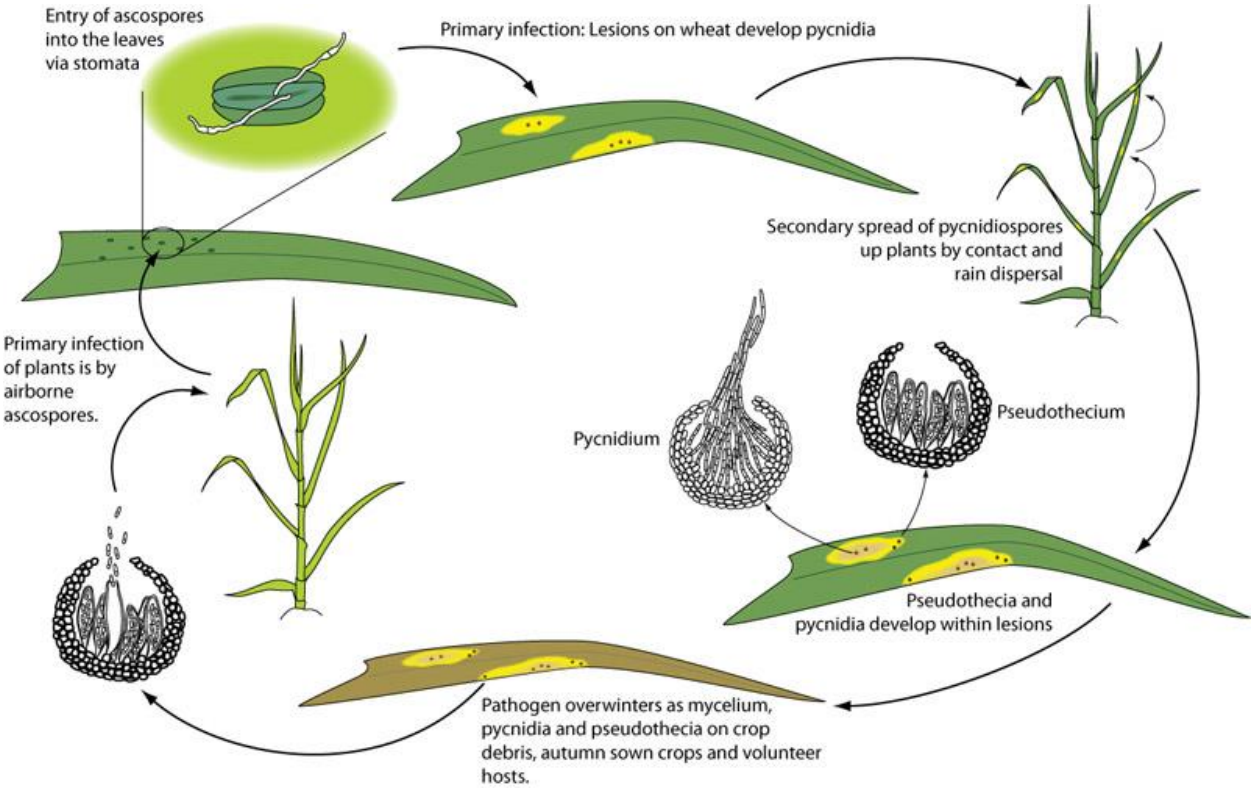


Figure 1.12 The lifecycle of *Z. tritici*. From Ponomarenko et al. 2011.

1.14 Controlling Septoria tritici blotch

There are two major ways of controlling STB in the field: fungicides and resistant hosts. Fungicides stop the fungus before infection can occur. The four classes of fungicides that have been used to control STB are the benzimidazoles, the QoIs (quinine outside inhibitors), the DMIs (demethylation inhibitors), and SDHIs (succinate dehydrogenase inhibitors).

The other method of control is resistant hosts. There are a variety of resistance genes found in wheat, and their durability varies (Ponomarenko et al. 2011). Resistance to *Z. tritici* is primarily found via quantitative resistance, however, many qualitative resistance genes have been found. These genes only protect wheat against a limited population of *Z. tritici* (Brown et al. 2015). Two of these genes have demonstrated gene-for-gene interactions with the competeing avirulence genes found in the *Z. tritici* genome; the Stb6 and Stb7 resistance genes from wheat, with the competing avirulence genes AvrStb6 and Avr3D1 respectively (Kema et al. 2000; Brading et al. 2002; Zhong et al. 2017; Meile et sal. 2018). Many studies have performed QTL mapping of quantitative resistance in *T. aestivum* and *T. turgidum* ssp. *durum*, and the results suggest that quantitative

resistance to STB is a complicated matter (Kema and Van Silfhout 1997; Eriksen et al. 2003; Simón et al. 2004; Tabib Ghaffary et al. 2011; Risser et al. 2011; Kelm et al. 2012; Goudemand et al. 2013; Mergoum et al. 2013; Miedaner et al. 2013; Stewart and McDonald 2014; Radecka-Janusik and Czembor 2014; Brown et al. 2015; Arraiano and Brown 2016; Stewart et al. 2017; Stadlmeier et al. 2019). With both forms of resistance, the best course of action seems to be to combine multiple types of resistance and resistant cultivars, as both qualitative and quantitative forms of resistance are likely to degrade over time and become less effective (McDonald and Mundt 2016; Borg et al. 2018; Kristoffersen et al. 2020).

The current state of control of STB is unstable. This pathogen evolves very quickly due to large field populations and cycles of sexual reproduction in the field. By the end of a growing season, up to 30% of the fungal population is the result of sexual reproduction (Zhan et al. 1998; Eriksen et al. 2001). This allows for rapid evolution and spread of fungicide resistance. Widespread resistance to the benzimidazoles was found in 1985, and resistance to the QoIs was first found in the cytochrome B gene in the UK in 2002 (Griffin and Fisher 1985; Fraaije et al. 2003). In the case of the QoI fungicides, it has been shown that both ascospores and sexual reproduction are important for the spread of resistance (Fraaije et al. 2005; Kema et al. 2018). Since it was first discovered, the allele for QoI resistance has been found worldwide, evolving independently in some cases (Estep et al. 2015; Neddaf et al. 2017; Cheval et al. 2017). The DMI fungicides are also starting to become less effective over time as they are used more frequently and different mutations accumulate in the cyp51 gene (Fraaije et al. 2007; Stammler et al. 2008; Estep et al. 2015). The last type of fungicide used to control STB are the SDHI fungicides. This class has been found to be at high risk of developing resistance, due to the number of amino acid changes that can reduce their efficacy (Fraaije et al. 2012). Unfortunately, resistance for this class has been found in the EU already (Dooley et al. 2016; Rehfus et al. 2018; Yamashita and Fraaije 2018). As such, the primary chemical methods of control are a mixture of DMI and SDHI fungicides. While the DMI and SDHI fungicides are currently effective, this is decreasing as resistance spreads.

While studies have investigated the use of biocontrol methods to control STB, they are not used in the field, and field trials generally have been inconclusive (Kildea et al. 2008; Perelló et al. 2009; McDonald and Mundt 2016; Samain et al. 2017; Latz et al. 2020). Alternate methods of control, whether chemical, biological, or cultural, are required to control this pathogen.

1.15 Zymoseptoria tritici and Light

As discussed earlier, light is a major environmental factor that controls many different aspects of a phytopathogenic fungus' life cycle and reproduction. Currently, little is known about how *Z. tritici* responds to light. The genome encodes all of the major light -sensing and -responsive genes found in Ascomycota, including the UV-specific endonuclease used in nucleotide excision repair (Goodwin et al. 2011; Tiley et al. 2019). It has been shown that *Z. tritici* can sense and respond to light (Tiley et al. 2019; McCorison and Goodwin 2020). *Z. tritici* shows some differential growth between day:night cycles and constant darkness (Tiley et al. 2019). Currently, VeA and VelB have been characterized by knockout mutations (Choi and Goodwin 2011; Tiley et al. 2019). VeA (MVE1) controls melanization, aerial hyphae production, response to osmotic stress, and hydrophobicity of the cell wall (Choi and Goodwin 2011). VelB is required for dimorphic growth, specifically the transition from filamentous to yeast-like growth, light-responsive growth, normal pycnidia formation and pycniospore production (Tiley et al. 2019).

While we do not know the mechanism of the primary light-sensing genes in *Z. tritici*, and the functions they have do not always translate from species to species, light responses in other fungi in the Capnodiales have been characterized and studied. In *Cercospora zeae-maydis*, light is very important for pathogenicity (Kim et al. 2011a). Light and the white collar 1 homolog (WC1) are required for the formation of appressoria, and WC1 is also required for stomatal tropism, production of cercosporin, and survival after exposure to UV radiation (Kim et al. 2011a). *C. kikuchii* uses circadian rhythm to control hyphal melanization, forming melanized rings as the fungus grows outward (Bluhm et al. 2010). *C. sorghi* and *C. beticola* also show a circadian rhythm, but not *C. zeae-maydis* or *C. zeina*, indicating that while a circadian rhythm can be found in the Capnodiales, it is not consistent (Bluhm et al. 2010). Light is required for full virulence in some Capnodiales species, such as *Mycosphaerella musicola* and various *Cercospora* species (Thorold 1940; Echandi-Zürcher 1959; Calpouzos and Corke 1963; Calpouzos 1966; Daub and Ehrenshaft 2000). Multiple Capnodiales species have photosensitizing toxins, that utilize light to produce ROS, including cercosporin in *Cercospora* species, and DHN melanin in *M. fijiensis*. The variety of important light responses observed in the few species that have been studied indicates that a hypothesis that light is important to the growth and pathogenicity of *Z. tritici* is valid and warrants further testing.

CHAPTER 2. THE WHEAT PATHOGEN ZYMOSEPTORIA TRITICI SENSES AND RESPONDS TO DIFFERENT WAVELENGTHS OF LIGHT

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2.1 Background

Light is essential for many biological processes and is an important environmental cue. Fungi have multiple responses to light, which can vary from species to species. Much of the research done on light responses in ascomycetes has been with the model filamentous fungus *Neurospora crassa* (Sargent and Briggs 1967; Harding and Turner 1981; Degli-Innocenti and Russo 1984; Nelson et al. 1989; Schmidhauser et al. 1990; Lauter and Russo 1991; Arpaia et al. 1995; Dasgupta et al. 2015; Wang et al. 2016a). This species has clearly delineated morphology between growth that occurs in the light versus the dark (Sargent and Briggs 1967; Harding and Turner 1981; Degli-Innocenti and Russo 1984; Nelson et al. 1989; Schmidhauser et al. 1990; Lauter and Russo 1991).

Multiple species in a variety of families rely on light to regulate the machinery that helps them cope with stresses. UV radiation damages DNA, and the cells respond by producing photolyase proteins to repair the damage. The expression of photolyases is often induced by light, as it is only required after exposure to UV (Berrocal-Tito et al. 1999; Alejandre-Durán et al. 2003; Bluhm and Dunkle 2008; Veluchamy and Rollins 2008; Brancini et al. 2018). Oxidative stress, which can be caused by light, relies on light-sensing genes to induce production of proteins required for cellular repair (Canessa et al. 2013; Wu et al. 2014). Many fungi are protected from photobiological damage by pigments, such as melanin and various carotenoids, production of which can be induced by light (Harding and Turner 1981; Ruiz-Roldán et al. 2008; Bluhm et al. 2010; Fuller et al. 2013; Wu et al. 2014; Krobanan et al. 2019; Tang et al. 2020).

Light can have major effects on fungal growth and morphology. In some species of fungi, light induces asexual reproduction via the formation and germination of conidia, and represses sexual reproduction (Mooney and Yager 1990; Blumenstein et al. 2005; Wang et al. 2016c, 2017b; Brandhoff et al. 2017; Suzuki et al. 2017). Yet the opposite pattern is found in other species

(Amaike and Keller 2009; Bluhm et al. 2010; Arazoe et al. 2015; Arraiano and Brown 2016). Growth in day:night cycles can lead to fungal colonies showing concentric circles of differing morphologies on agar plates (Das and Busse 1990; Bluhm et al. 2010; Hubballi et al. 2010). Presumably this also would occur within plant hosts and may explain the spread of lesions in concentric circles seen in fungi such as many species in the genus *Alternaria*, some of which show maximum growth in culture under alternating cycles of light and dark (Hubballi et al. 2010).

In some fungal species, light regulates when toxins and other secondary metabolites are produced (Kale et al. 2008; Duran et al. 2009; Amaike and Keller 2009; Kim et al. 2011b; Fanelli et al. 2012b, 2017; Suzuki 2018). In *Cercospora* species that produce the light-activated phytotoxin cercosporin, light induces production of the toxin, as that is when it is most effective (Daub and Ehrenshaft 2000; You et al. 2008), and no cercosporin is produced in the dark. A similar phenomenon occurs in *Alternaria alternata* where production of the mycotoxins altertoxin and alternariol are induced exclusively or have expression increased greatly under blue light in contrast to dark (Pruß et al. 2014). Similarly, the production of aflatoxin in *Aspergillus* species can be affected by both the color and intensity of light, although the conditions under which mycotoxins are produced at the highest rates are not uniform within the genus (García-Cela et al. 2015; Suzuki 2018). Alternatively, in *Fusarium graminearum*, light represses the production of the trichothecene mycotoxins deoxynivalenol and 15-acetyl-deoxynivalenol (Kim et al. 2014a).

Curiously, the ability to sense light also is required for pathogenicity and virulence in some pathogenic fungal species. For example, in the plant pathogens *Botryis cinerea* and *Cercospora zea-maydis*, knocking out *wc1*, the light-sensing component of the proteins comprising the White Collar Complex (WCC), leads to lowered virulence or a complete lack of pathogenicity (Kim et al. 2011a; Canessa et al. 2013). In *Aspergillus flavus*, the deletion of *wc1* or *velvet* homologs drastically reduce the ability of the fungus to infiltrate corn kernels, peanuts, and cotton bolls (Kale et al. 2008; Duran et al. 2009; Amaike and Keller 2009). However, in the rice pathogen *Magnaporthe oryzae*, light represses infection, and leads to much lower disease severity (Kim et al. 2011c). A *wc1* knockout in *M. oryzae* showed greater disease severity, as it could infect in the light as well as in the dark, compared to wild type which only infects in the dark (Kim et al. 2011c).

Zymoseptoria tritici (synonyms: *Mycosphaerella graminicola, Septoria tritici*) is the causal agent of Septoria tritici blotch, an economically important disease of wheat. Losses due to this disease can reach up to 50% in epidemic years, and often vary between 5 - 20% depending on the

environment and the cultivar of wheat; it has been estimated that up to 70% of fungicide use in Europe is to control this disease (Eyal et al. 1987; Fones and Gurr 2015; Torriani et al. 2015). Spores of *Z. tritici* are splash dispersed during rainstorms, and need humid conditions for successful infection (Shaw and Royle 1993). After landing on a leaf, the spores germinate, and invade the wheat plants via the stomata (Kema et al. 1996). Initial growth appears to be biotrophic, but the fungus rapidly switches to necrotrophic growth beginning 12-14 days after penetration (Ponomarenko et al. 2011). Controlling *Z. tritici* is becoming more difficult, as resistance to the strobilurin (quinone-outside inhibitor) fungicides has become widespread in Europe, and also has appeared in North America (Torriani et al. 2015; Cheval et al. 2017). Other effective fungicides are the demethylation inhibitors (azoles), the SDHIs (succinate dehydrogenase inhibitors), and multi-site fungicides (Torriani et al. 2015). Resistance to the azoles has already begun to spread, and the SDHI fungicides are at a medium to high risk of fungal populations developing resistance (Fraaije et al. 2012; Estep et al. 2015; Torriani et al. 2015; Wieczorek et al. 2015; Rehfus et al. 2018). Understanding more about how *Z. tritici* infects wheat and what conditions are necessary for it to reproduce are critical for developing better methods of disease control.

Currently, little is known about how *Z. tritici* senses and responds to light. Light does have minor effects on the growth and development of *Z. tritici*, stimulating formation of aerial hyphae, and prolonging the time growing in yeast-like form before transitioning to hyphal form (Choi and Goodwin 2011; Tiley et al. 2019). Light is a very important environmental cue for some fungi in the Dothideomycetes, the class that contains *Z. tritici* and many other important plant pathogens (Quaedvlieg et al. 2011; Ohm et al. 2012). For example, in the genus *Cercospora*, another genus in the same taxonomic order (Capnodiales), light represses asexual sporulation, and melanization is controlled by circadian rhythms (Bluhm et al. 2010; Kim et al. 2011a). The disruption of *CRP1*, a homolog of *wc1* in *N. crassa*, eliminates the stomatal tropism that *C. zea-maydis* needs to infect maize (Kim et al. 2011a). The conidiation and growth of two species in the order Pleosporales, *Alternaria alternata* and *Exserohilum turcicum*, are also regulated by light (Flaherty and Dunkle 2005; Pruß et al. 2014). Additionally, as mentioned previously, light regulates toxin production in multiple Dothideomycetes species (Daub et al. 2005; You et al. 2008; Pruß et al. 2014; Fanelli et al. 2017).

The *Z. tritici* genome contains genes coding for a number of homologs to photoreceptors in other fungi (Goodwin et al. 2011). These include homologs for the two White Collar Complex

genes (*wc1* and *wc2*), VIVID, a blue-light-sensing cryptochrome, a photolyase plus two putative photolyase genes and a red-light-sensing phytochrome (Blumenstein et al. 2005; Bluhm and Dunkle 2008; Smith et al. 2010; Gin et al. 2013). It also has homologs of some genes that may not be involved in sensing light, but do respond to light in other species, such as *frq*, a circadian rhythm gene that codes for the frequency clock protein, and *velvet*, which responds to light and has been shown to react with a phytochrome and the white collar complex in *Aspergillus nidulans* (Froehlich et al. 2002; Lombardi and Brody 2005; Hunt et al. 2010; Bayram and Braus 2012). Knocking out function of the velvet gene, *MVE1*, in *Z. tritici* had large effects on growth and development, including increased sensitivity to stresses, reduced melanin production, and blindness to light-induced aerial hyphae formation (Choi and Goodwin 2011).

Between the evidence that closely related fungi can sense light and the presence of putative photoreceptors and photoreceptor homologs, it seems highly likely that *Z. tritici* can sense and respond to light, but this has not been tested specifically. The goal of this research was to test the hypothesis that *Z. tritici* can sense and respond to light. Secondary goals were to identify genes that are highly regulated by light for future study and to augment the annotation of the reference genome by analyzing the expression of genes induced by different wavelengths of light.

2.2 Results

2.2.1 RNA sequencing

RNA samples extracted from mycelia grown in three biological replicates of four light growth conditions, 16 hours per day of white, blue (400-530 nm), or red (600-700 nm) light and continuous darkness, were sequenced and the poly A, unstranded reads were filtered for quality, mapped, and analyzed. On average, 1.35% of all sequence reads were too short after trimming and were removed from each of the samples, and an average of 98.7% of the reads mapped to the *Z. tritici* genome annotations (Table 2.1). The remaining 1.3% of the reads mostly appeared to be contaminants. When the read counts for each gene were plotted between the three biological replicates of each condition, the reproducibility was very high (Figure 2.1A). The lowest variability appeared to occur between the three white light replicates, while the greatest was between the first and third dark replicates (Figure 2.1A); however, even this was low compared to the variability among the different treatments.

Table 2.1 Summary statistics about RNA sequence reads and mapping to the reference genome for *Zymoseptoria tritici* cultures exposed to different light treatments.

Treatment	Replicate	Total raw reads (millions)	Reads mapped to reference genome (millions)	Percentage of reads mapped to reference genome
White light	1	32.50	31.32	97.8
	2	45.33	41.32	92.7
	3	40.20	38.72	97.8
Blue light	1	42.21	40.72	97.9
	2	32.52	31.33	97.8
	3	55.45	53.49	97.9
Red light	1	44.38	42.86	98.0
	2	43.32	41.65	97.7
	3	36.64	35.41	98.1
Dark	1	156.64	154.20	99.2
	2	114.25	112.89	99.7
	3	152.06	150.29	99.7

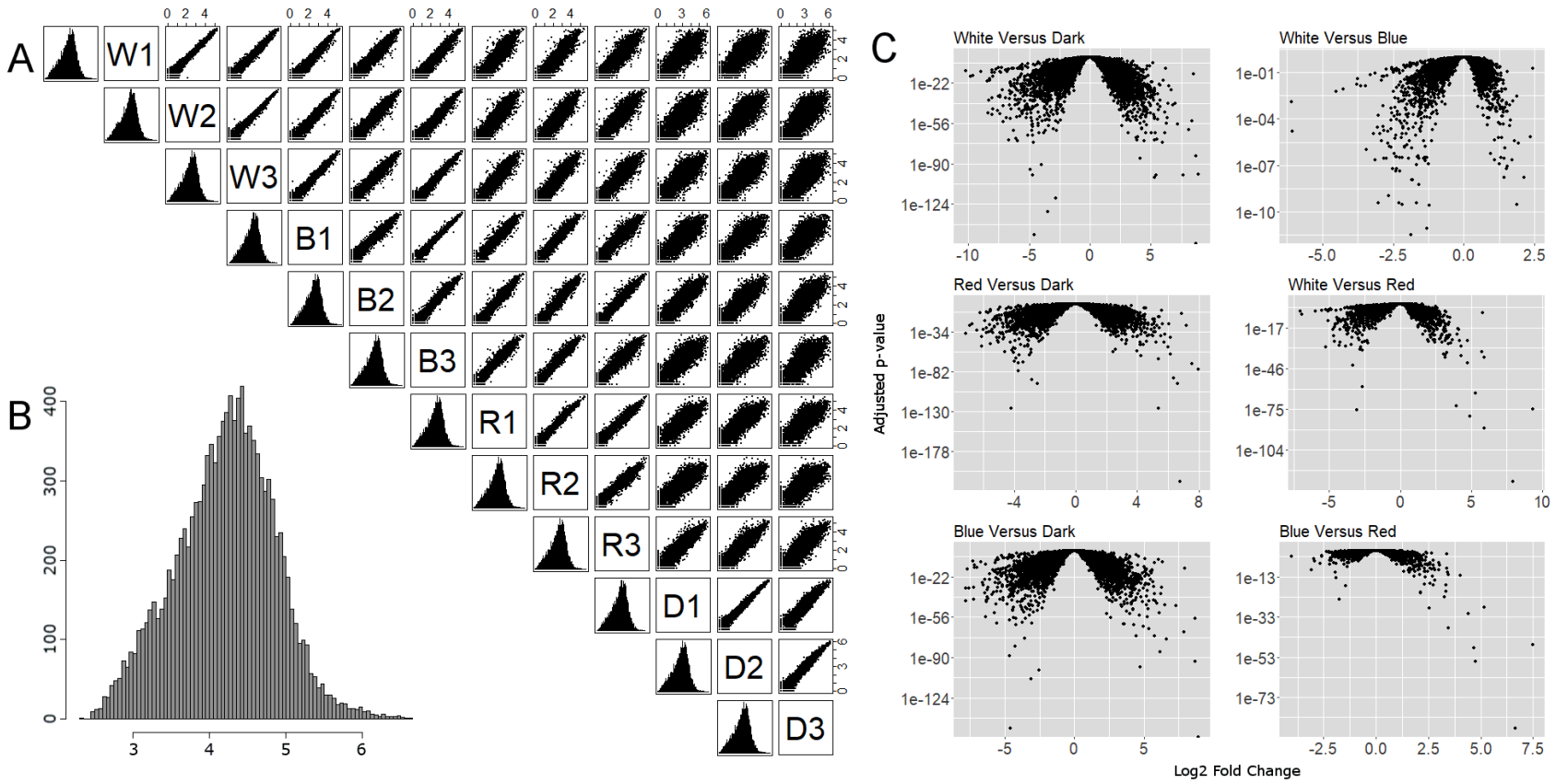


Figure 2.1 Quality-control checks of the read libraries. A, To the right of the treatment diagonal: Scatterplots comparing two replicates on a log10 scale; To the left of the treatment diagonal: Individual histograms showing the gene read count distribution over each replicate. Treatments of white, blue and red light, and dark are indicated in the diagonal by W, B, R and D, respectively. Replication number is indicated by an integer from 1-3, e.g., W2 is the second replication of the white light treatment. B. Histogram of the gene read sums across all replicates. The x axis is the log10 read count and the y axis is the number of genes. C. Volcano plots of the log2 fold changes versus the adjusted p values

Out of 13,522 total genes called in the reference genome, 12,079 (89%) had greater than 10 reads in each replicate and more than 100 reads across all twelve replicates, and were used in analysis with DESeq2. The cleaned data were distributed normally, both within individual replicates and as a whole (Figure 2.1B).

2.2.2 Differential gene expression in response to light

There were major differences in the numbers of differentially expressed genes (adjusted p value < 0.05 and an absolute log2 fold change (LFC) > 2) between the dark condition and any of the three light treatments, with all three of the light conditions versus dark having over 2000 differentially expressed genes each, while white versus red was the only other comparison to break 100 differentially expressed genes (Table 2.2). The white light versus red light comparison was distinct from the white light versus blue light and red light versus blue light comparisons, as well as from the light vs dark comparisons (Table 2.2). All six comparisons between the treatments showed the expected concavity in the volcano plots, but the magnitudes of the adjusted p-values were much greater in the three light versus dark comparisons, with the lowest magnitude of adjusted p-values in white light versus blue light (Figure 2.1C).

Table 2.2 Differentially expressed genes between cultures of *Zymoseptoria tritici* exposed to dark or different wavelengths of light.

Criterion[a]	White vs dark	Blue vs dark	Red vs dark	White vs blue	White vs red	Blue vs red
LFC 2 Up	1524	1334	1207	4	224	72
LFC 2 Down	1900	1527	1296	140	563	24
Total LFC 2	3424	2861	2503	144	787	96
% annotated[b]	51.6	50.3	48.9	36.8	39.5	45.8

a Criteria were adjusted p value < 0.05 and an absolute log2 fold change (LFC) > 2.
b % annotated indicates genes with functional annotations using GO, KEGG or EggNOG annotations.

In total, 4187 unique genes were differentially expressed between all six comparisons. This is just under a third (31%) of all genes present in the *Z. tritici* genome. In the three light versus dark comparisons, 4019 unique genes were differentially expressed, which is very near the total unique genes, and still represents 30% of all genes in *Z. tritici*. In the three light versus light

comparisons, 804 unique genes were differentially expressed, which is only about 6% of the genes in the *Z. tritici* genome (Additional File 2).

The dark condition had the highest number of unique differentially expressed genes in all three comparisons with the light treatments (Figure 2.2). Blue light consistently had the fewest unique differentially expressed genes compared to all of the other treatments (Figure 2.2). Many of the genes that are differentially expressed between any light condition and full darkness are differentially expressed between all light conditions and full darkness, with white light having the highest number of unique differentially expressed genes (Figure 2.2).

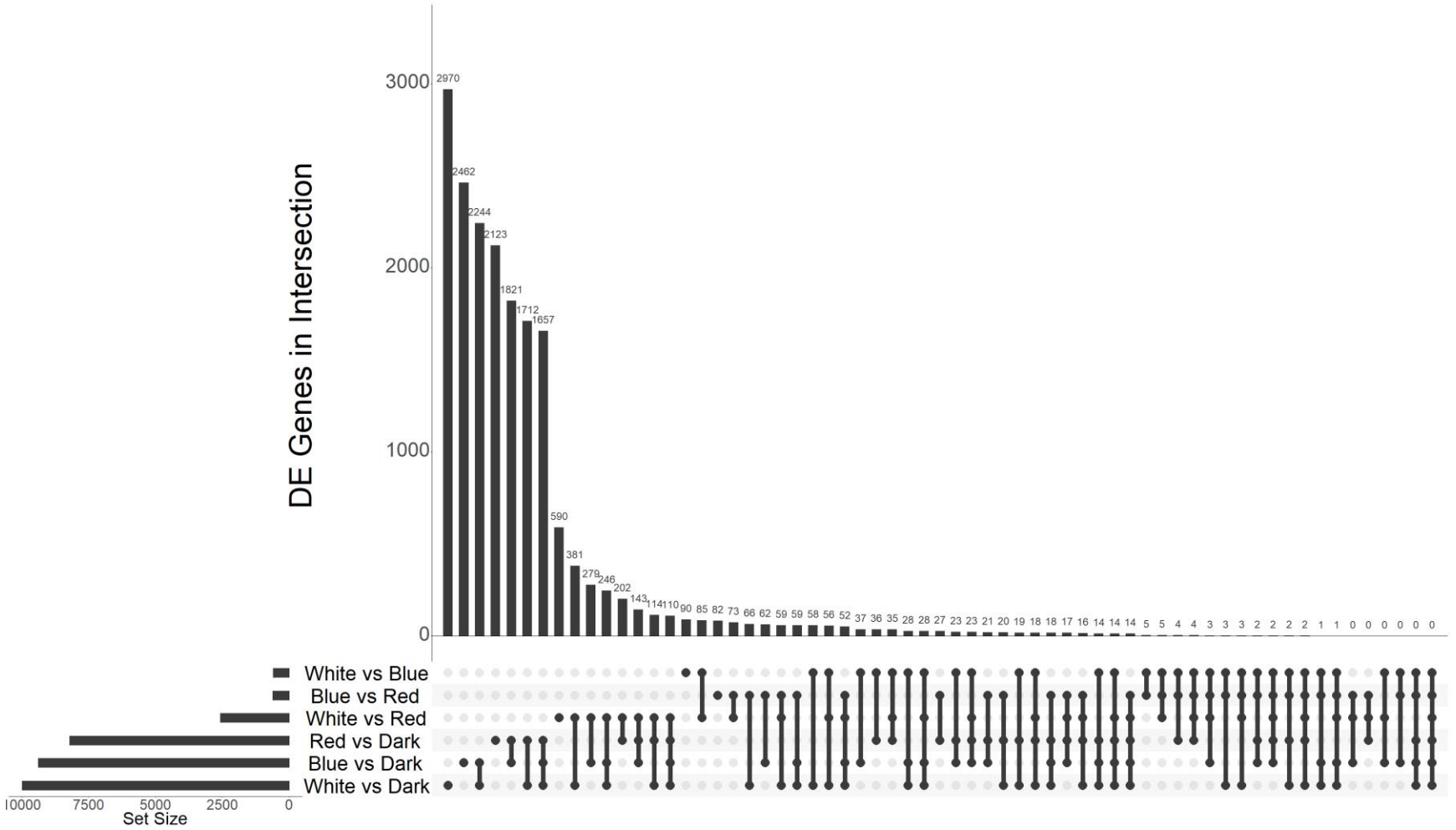
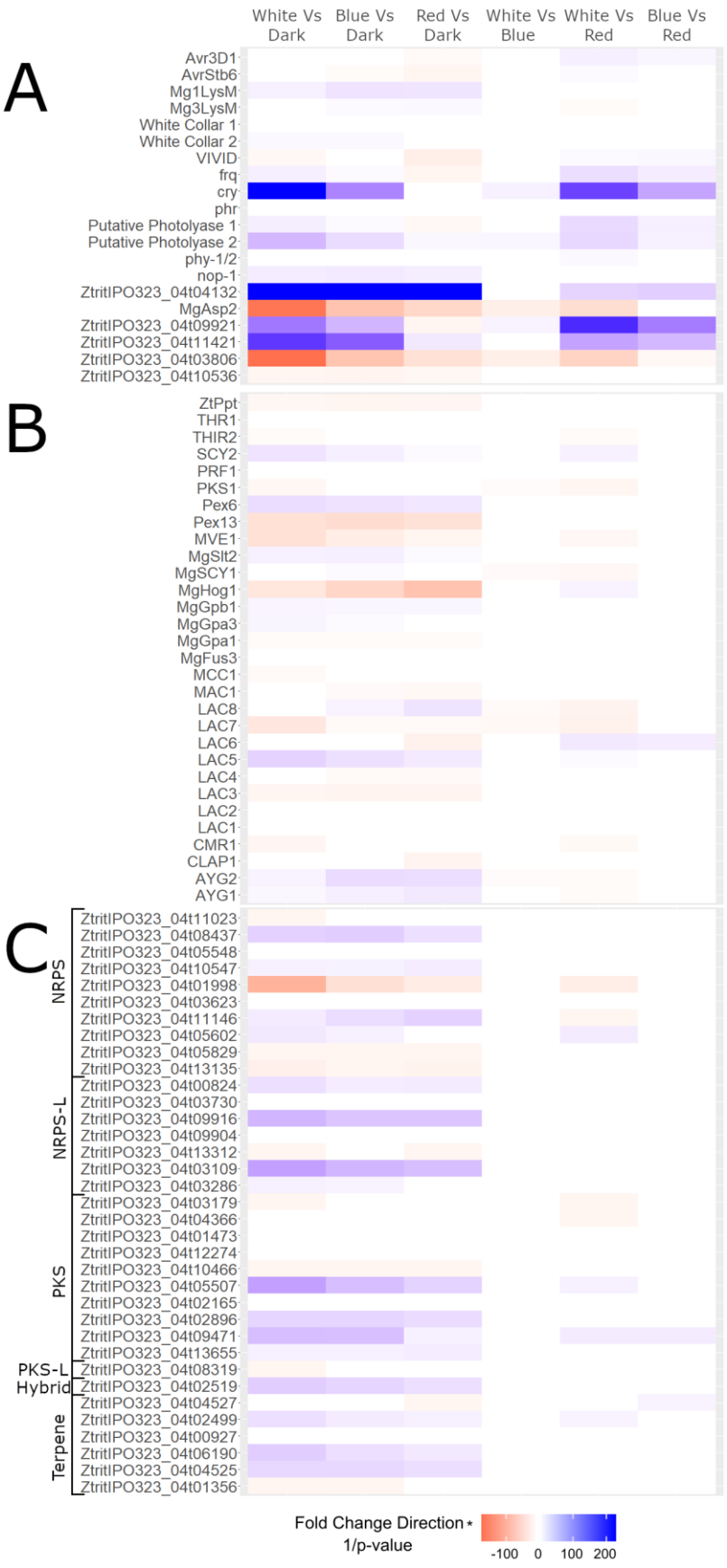


Figure 2.2 An UpSet plot showing the number of unique and shared differentially expressed genes of *Zymoseptoria tritici* between each comparison (Conway et al. 2017).

Receptors play an important role in sensing and responding to light. Many of the potential light-sensing proteins in *Z. tritici* were not differentially expressed in any comparison (Figure 2.3A). Multiple genes with photolyase-like regions were differentially expressed under various conditions. The cryptochrome/photolyase gene CRY was upregulated in white light and blue light, but not in red light or darkness. The rhodopsin-like gene NOP-1 was upregulated in all types of light versus dark. The known light-signaling gene MVE1 was upregulated in the dark relative to white light and blue light (Figure 2.3A). Interestingly, four genes for effector proteins were differentially expressed under different light conditions, two LysM effectors, Avr3D1, and AvrStb6 (Figure 2.3A).

There are three MAPK (mitogen-activated protein kinase)-encoding genes in *Z. tritici* (Cousin et al. 2006; Mehrabi et al. 2006b, a). *MgHog1* (ZtritIPO323_04g02798) was strongly downregulated in light, especially in red light (Figure 2.3B). Another MAPK-encoding gene, *MgSlt2* (ZtritIPO323_04g00461), was upregulated in light (Figure 2.3B). A third MAPK-encoding gene, *MgFus3* (ZtritIPO323_04g10805), was not differentially expressed in any comparison (Figure 2.3B). All three of these MAPK-encoding genes are essential for full virulence of *Z. tritici* as well as the production of melanin (Cousin et al. 2006; Mehrabi et al. 2006b, a), a critical component of photoprotection in this fungus. Another melanization-related gene, *PKS1*, is downregulated in white light versus blue and red light, which indicated that it also may be regulated by a light color such as green, or by an interplay of the blue-light and red-light sensing genes (Figure 2.3B) (Lendenmann et al. 2014).

Figure 2.3 A heatmap of modified adjusted p values for selected *Zymoseptoria tritici* genes possibly involved in pathogenicity and light sensing and response. The values are the inverse of the adjusted p value multiplied by the direction of the fold change. A. Genes for light sensing, known effector and AVR genes, and selected differentially expressed genes from Additional File 3. B. Genes related to melanin production. C. Genes related to secondary metabolism genes (NRPS: Non-ribosomal peptide synthetases, NRPS-L: NRPS-like, PKS: Polyketide synthase, PKS-L: PKS-like.)


White Vs Dark
Blue Vs Dark
Red Vs Dark
White Vs Blue
White Vs Red
Blue Vs Red
A
Avr3D1
AvrStb6
Mg1LysM
Mg3LysM
White Collar 1
White Collar 2
VIVID
frq
cry
phr
Putative Photolyase 1
Putative Photolyase 2
phy-1/2
nop-1
ZtritIPO323_04t04132
MgAsp2
ZtritIPO323_04t09921
ZtritIPO323_04t11421
ZtritIPO323_04t03806
ZtritIPO323_04t10536
B
ZtPpt
THR1
THIR2
SCY2
PRF1
PKS1
Pex6
Pex13
MVE1
MgSlt2
MgSCY1
MgHog1
MgGpb1
MgGpa3
MgGpa1
MgFus3
MCC1
MAC1
LAC8
LAC7
LAC6
LAC5
LAC4
LAC3
LAC2
LAC1
CMR1
CLAP1
AYG2
AYG1
C
NRPS
NRPS-L
PKS
PKS-L Hybrid
Terpene
ZtritIPO323_04t11023
ZtritIPO323_04t08437
ZtritIPO323_04t05548
ZtritIPO323_04t10547
ZtritIPO323_04t01998
ZtritIPO323_04t03623
ZtritIPO323_04t11146
ZtritIPO323_04t05602
ZtritIPO323_04t05829
ZtritIPO323_04t13135
ZtritIPO323_04t00824
ZtritIPO323_04t03730
ZtritIPO323_04t09916
ZtritIPO323_04t09904
ZtritIPO323_04t13312
ZtritIPO323_04t03109
ZtritIPO323_04t03286
ZtritIPO323_04t03179
ZtritIPO323_04t04366
ZtritIPO323_04t01473
ZtritIPO323_04t12274
ZtritIPO323_04t10466
ZtritIPO323_04t05507
ZtritIPO323_04t02165
ZtritIPO323_04t02896
ZtritIPO323_04t09471
ZtritIPO323_04t13655
ZtritIPO323_04t08319
ZtritIPO323_04t02519
ZtritIPO323_04t04527
ZtritIPO323_04t02499
ZtritIPO323_04t00927
ZtritIPO323_04t06190
ZtritIPO323_04t04525
ZtritIPO323_04t01356
Fold Change Direction *
1/p-value
-100
0
100
200

Three small, secreted proteins (SSP) are highly differentially expressed in various comparisons (Figure 2.3A) (Palma-Guerrero et al. 2017). ZtritIPO323_04g09921 is strongly upregulated in blue and white light versus red light and dark and is downregulated in red light versus dark. This indicated that expression of this gene might be increased by white and blue light but suppressed by red light relative to dark. Another SSP, ZtritIPO323_04g11421, was highly upregulated in blue and white light versus red light and dark, indicating that its expression may be stimulated by the blue light spectrum. The third SSP, ZtritIPO323_04g03806, is strongly downregulated in all light conditions versus darkness, so may be repressed by all types of light. All three of these SSPs are functionally unannotated, and had no fungal matches in the NCBI NR database. One of the few SSPs with a functional annotation, ZtritIPO323_04g10536, has a non-orthologous group (NOG) of "chitin binding peritrophin-A" domains (10PPR@NOG according to the eggNOG annotation) (Huerta-Cepas et al. 2016), and is slightly downregulated in light (log2 fold change in white versus dark: -2.16, blue versus dark: -2.01, red versus dark: -1.88).

One protease had a highly significant expression pattern (Figure 2.3A) (Palma-Guerrero et al. 2017). MgAsp2 (ZtritIPO323_04g06056) was downregulated in all but one comparison, the exception being blue versus red light. The NOG associated with this gene is 03JP4@ascNOG, a secreted aspartic protease orthologous group, and blastp links it to other aspartic proteases (Huerta-Cepas et al. 2016). Many other proteases had significant differences in transcript abundance between treatments, with approximately a third of the proteases upregulated, a third downregulated, and a third not differentially expressed in the light versus dark comparisons (Figure 2.4D).

No obvious patterns were apparent in differential expression of genes for secreted proteins from other functional classes. Very few secreted lipase genes were differentially expressed in the various light versus dark comparisons (Figure 2.4B). Most secreted peroxidases were more differentially expressed in white light rather than blue or red (Figure 2.4E). Almost half of the plant cell wall degrading enzymes (PCWDEs) were downregulated by light, but with no consistent pattern (Figure 2.4C).

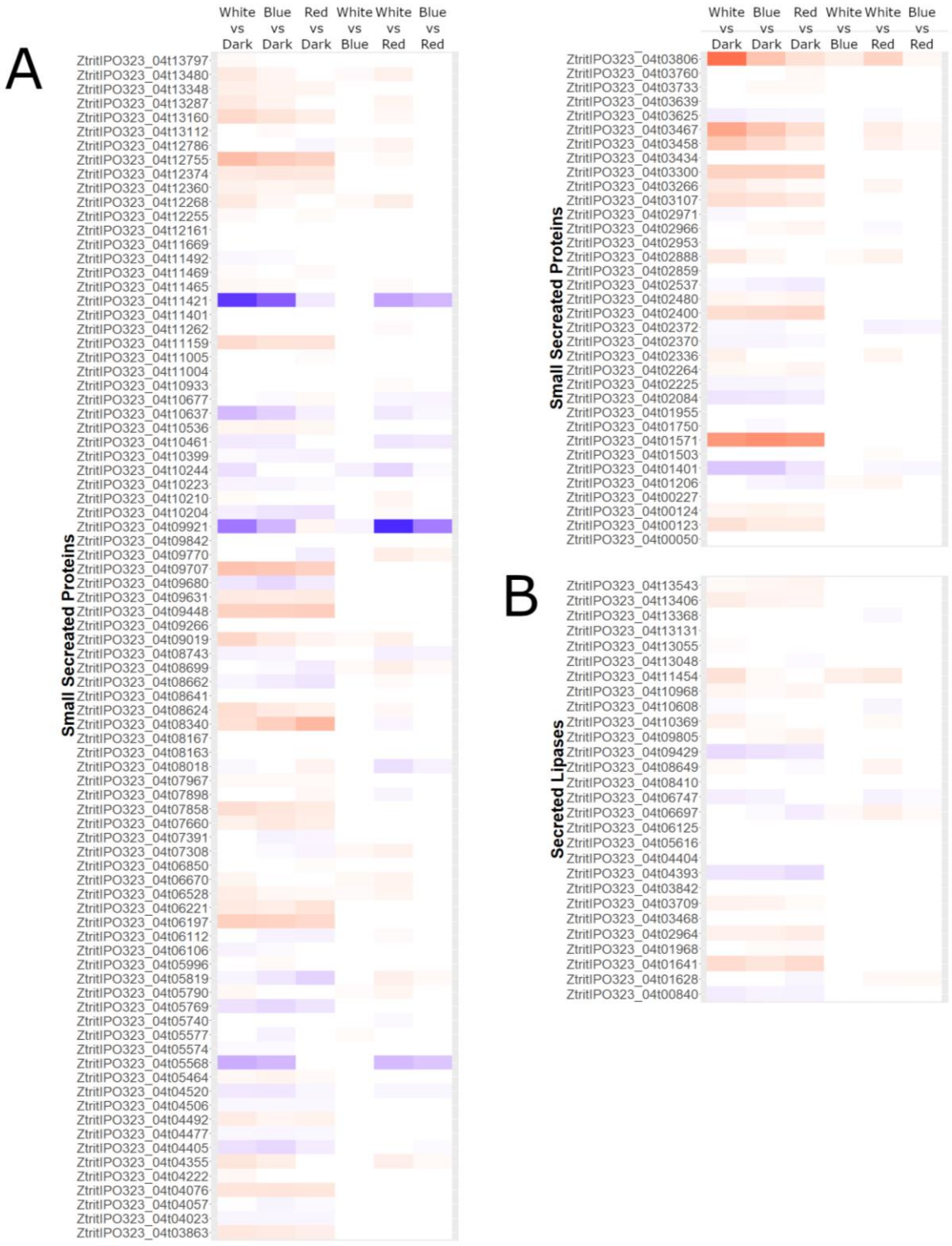
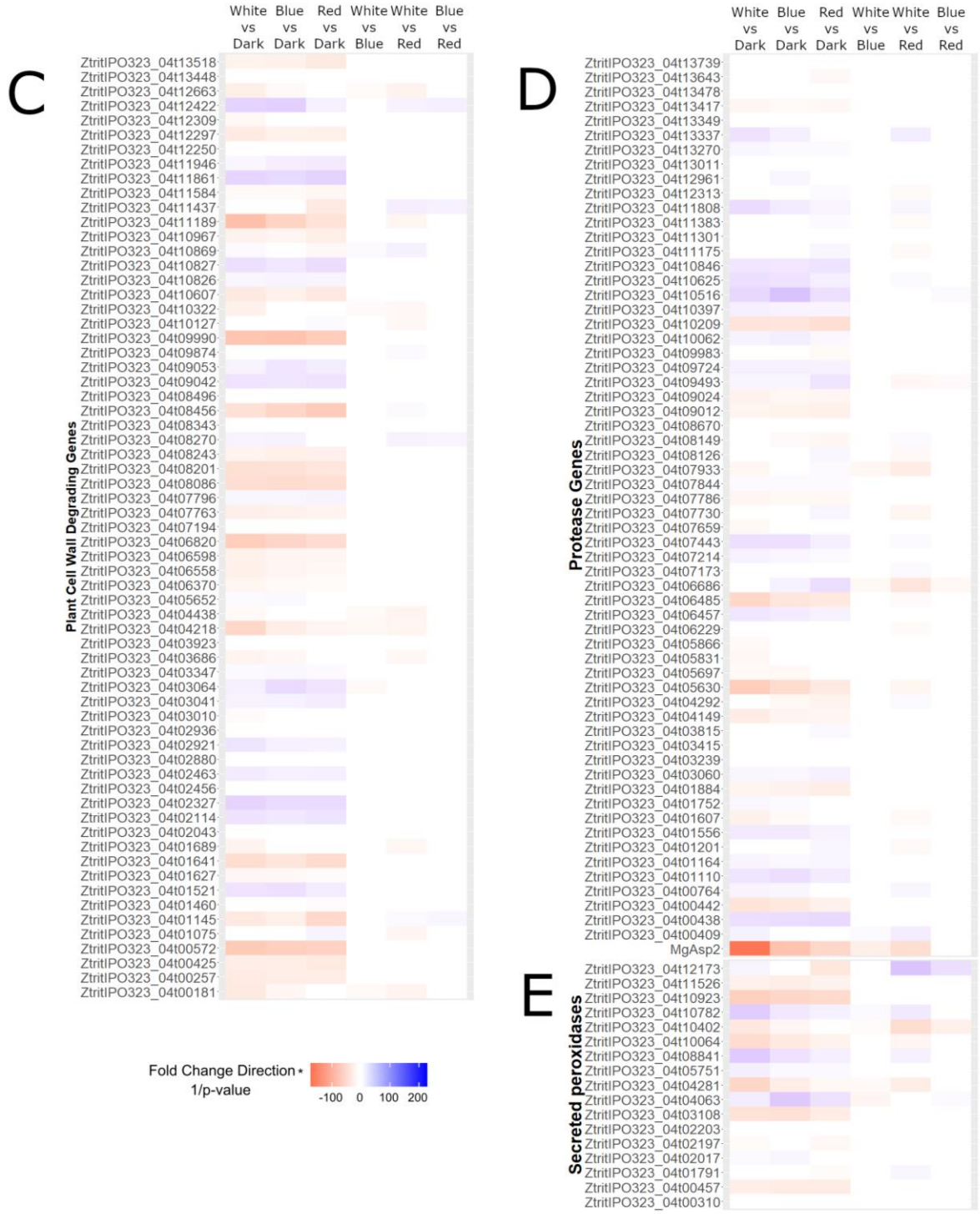


Figure 2.4 Heatmaps of modified adjusted p values for selected *Zymoseptoria tritici* genes possibly involved in pathogenicity and light sensing and response. The values are the inverse of the adjusted p value multiplied by the direction of the fold change. Comparisons are indicated at the top of each column. The classes A-E are from Palma-Guerrero et al. (Palma-Guerrero et al. 2017)

Figure 2.4 continued



The genes related to secondary metabolism (Cairns and Meyer 2017) tend to be upregulated by light if they are differentially expressed. Among all the genes, nearly half of them are upregulated, and only a quarter of them are downregulated in light versus dark comparisons (Figure 2.3C). In each of the categories of secondary metabolism genes, most had more genes upregulated than downregulated or not differentially expressed (Figure 2.3C). The exception is the polyketide synthase-like genes (PKS-L), where there was only one, and it was downregulated only in white light versus dark (Figure 2.3C).

Gene ZtritIPO323_04g04132 showed very high differential expression (Figure 2.3A). The adjusted p value for this gene in the white versus dark and blue versus dark comparisons was 0, with a log2 fold change of 8.7 and 8.9, respectively, and it had an adjusted p value of 1.1e-214 and a log2 fold change of 6.7 in the red versus dark comparison. It was also less dramatically differentially expressed in comparisons between white light versus red and blue versus red. The only functional annotations for this gene are an alpha-beta hydrolase fold (03NR3@ascNOG) and a methyl ester carboxylesterase conserved domain according to the NCBI Conserved Domain Database; it was not annotated as being in a class related to pathogenicity by Palma-Guerrero et al. (Marchler-Bauer and Bryant 2004; Marchler-Bauer et al. 2011, 2015, 2017; Huerta-Cepas et al. 2016; Palma-Guerrero et al. 2017).

Among all differentially expressed genes, approximately 3% of those in the light treatments relative to dark were located on dispensable chromosomes that are not present in all isolates of the pathogen (Goodwin et al. 2011); all of the rest occurred on the core chromosome set. Very few genes on dispensable chromosomes were differentially expressed when the three light treatments were compared to each other, ranging from 1 in the blue versus red light comparison to 57 between white light and red (Table 3).

Table 2.3 Numbers of differentially expressed genes on core (numbers 1-13) and dispensable (14-21) chromosomes in the six comparisons of RNA sequences from Zymoseptoria tritici cultures exposed to different wavelengths of light or kept in the dark.

Chromosome	White versus dark	Blue versus dark	Red versus dark	White versus blue	White versus red	Blue versus red	Total genes
1	548	433	368	6	100	14	2319
2	327	272	233	12	46	2	1393
3	286	235	194	6	67	15	1307
4	244	196	181	7	48	7	998
5	239	196	178	6	43	6	987
6	202	175	144	8	41	5	829
7	189	155	129	9	48	8	822
8	183	156	130	3	26	5	848
9	155	136	110	5	33	2	714
10	148	127	110	2	22	5	616
11	146	129	114	4	24	3	605
12	107	87	77	1	14	3	521
13	95	76	54	3	21	6	423
14	16	13	7	0	3	0	159
15	12	8	9	6	15	1	142
16	12	16	21	2	5	0	164
17	6	3	11	7	15	0	131
18	30	30	25	0	0	0	130
19	13	12	13	1	7	0	139
20	7	3	6	2	9	0	129
21	5	4	9	0	3	0	114
Core 1-13	2869	2373	2022	72	533	81	12382
Dispensable 14-21	101	89	101	18	57	1	1108
Total	2970	2462	2123	90	590	82	13490
Percent dispensable	3.4	3.6	4.8	20.0	9.7	1.2	8.2

2.2.3 Functional Characteristics

From 48-63% of the genes with significant differential expression in each comparison had no corresponding functional annotation (Table 2.2). This includes KOG classes and GO terms in the frozen gene catalog created as part of the original annotation by the Joint Genome Institute (Goodwin et al. 2011), as well as EggNOG classifications (Huerta-Cepas et al. 2016) generated with the analysis of additional RNA sequences by King (King). On average, 45.5% of genes could be assigned functional protein annotations, while 27.7% could be assigned GO terms. In the full genome, 55.3% of genes have annotations, and 32.8% have GO terms assigned. There is a distinct division between the three light/dark comparisons and the light/light comparisons; the light/light comparisons have a higher percentage of genes annotated to the full genome (average of 59.3%), while the light/dark comparisons have fewer genes annotated than the full genome (average of 49.7%).

The enriched GO terms were quite diverse between the comparisons. The numbers of GO terms enriched between the three light treatments and the dark treatment were higher than those between the light conditions (Table 2.4). This was similar to the raw numbers of differentially expressed genes (Table 2.2).

Table 2.4 Number of enriched Gene Ontology terms for the up- and down-regulated genes in each comparison

Direction of change	White versus dark	Blue versus dark	Red versus dark	White versus blue	White versus red	Red versus blue
Up-regulated	138	106	110	1	39	10
Down-regulated	126	177	89	118	148	62

In pairwise comparisons between the light conditions, white light had fewer enriched GO terms than blue or red (Table 2.4). Compared to blue, white light had only one enriched GO term, protein phosphorylation (GO:0006468), that was also enriched in the dark in all three of the light versus dark comparisons. The gene this GO term was associated with was a serine/threonine kinase. More enriched GO terms were identified when cultures were grown under blue or red light than in the wider-range, white-light condition.

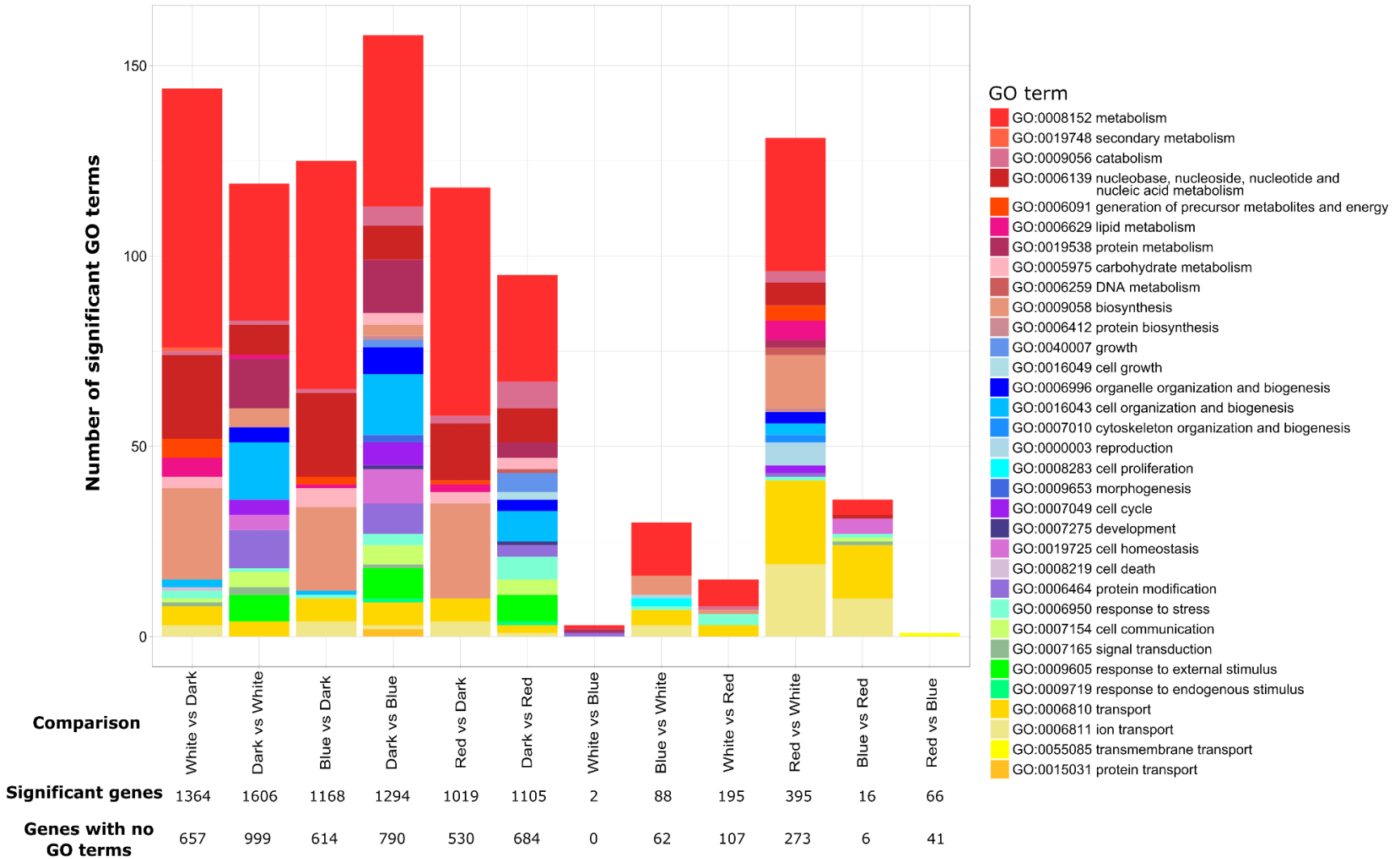
The dark condition had a wider range of enriched GO terms relative to the light conditions (Figure 2.3A). The variety of enriched GO terms was especially notable in the dark versus blue light comparison (Figure 3A). The dark treatment had more GO terms that were related to growth and development than did cultures grown in the light, where those GO terms occurred rarely. This was very apparent in the blue light and red light versus dark comparisons, where multiple GO terms for growth were enriched in the dark in each comparison. These GO terms included the high-level growth GO term (GO:0040007), as well as filamentous growth (GO:0030447), chromosome segregation (GO:0007059), cellular component biosynthesis (GO:0044085), and numerous cellular components related to mitotic division (GO:0000776, GO:0000794, GO:0000942, GO:0000793), among others.

The light conditions versus dark had many more enriched GO terms related to metabolism (Figure 2.5) as well as some that were related to transportation of substances in the cell. The dark versus white and dark versus blue comparisons were enriched in GO terms related to growth while the white versus dark comparison contained several GO terms related to communication.

The comparisons between the three light treatments had fewer enriched GO terms compared to the light versus dark comparisons. The exception to this was the genes that were downregulated in white versus red light, which looked more like a comparison with a dark treatment than did the white versus red and blue versus red comparisons (Figure 2.5).

Many of the light comparisons have enriched GO terms for responses to oxidative stress, including those that are enriched on both sides of a comparison, such as the general response to oxidative stress (GO:0006979), which was enriched in both white and red light. White and blue light had more of these GO terms enriched than red light, and dark had none. These include response to oxidative stress (GO:0006979), a response to hydrogen peroxide (GO:0042542), peroxidase activity (GO:0004601), and multiple oxidoreductase-related GO terms (GO:0016684, GO:0016705, GO:0016634, GO:0006733, GO:0016634, GO:0016722).

Figure 2.5 Stacked bar charts showing enriched Gene Ontology (GO) terms in comparisons between *Zymoseptoria tritici* transcriptomes after exposure to different light treatments. The first condition listed is upregulated, e.g., White vs Dark indicates GO terms upregulated in white light versus dark. The first six comparisons from left to right are light treatments compared to the dark, while the remaining six bar charts show comparisons between the different light treatments. Bars show the numbers of significant GO terms in each of the major categories indicated by color according to the legend on the right. Individual genes may have more than one GO term. The total number of differentially expressed genes and the number of those that had no GO terms for each comparison are indicated below the relevant bars.



Comparison	White vs Dark	Dark vs White	Blue vs Dark	Dark vs Blue	Red vs Dark	Dark vs Red	White vs Blue	Blue vs White	White vs Red	Red vs White	Blue vs Red	Red vs Blue
Significant genes	1364	1606	1168	1294	1019	1105	2	88	195	395	16	66
Genes with no GO terms	657	999	614	790	530	684	0	62	107	273	6	41

The KOG functional annotations had similar results to the GO annotations, showing that more genes related to the transport and metabolism of major substances are upregulated in the light rather than the dark. The class “defense mechanisms” also had more genes upregulated in the light than in the dark. The opposite trend occurred with some KOG classes related to growth, which had more genes upregulated in the dark. These KOG classes include “cell cycle control, cell division, chromosome partitioning”, “cell wall/membrane/envelope biogenesis”, “signal transduction mechanisms”, “replication, recombination and repair”, and “intracellular trafficking, secretion, and vesicular transport”. These differences did not appear in the light versus light comparisons. Despite these changes in transcription of genes involved in growth and development and previous studies (Choi and Goodwin 2011; Tiley et al. 2019), there were no obvious differences in the morphologies of cultures grown in the dark versus any of the light conditions (Figure 2.6).

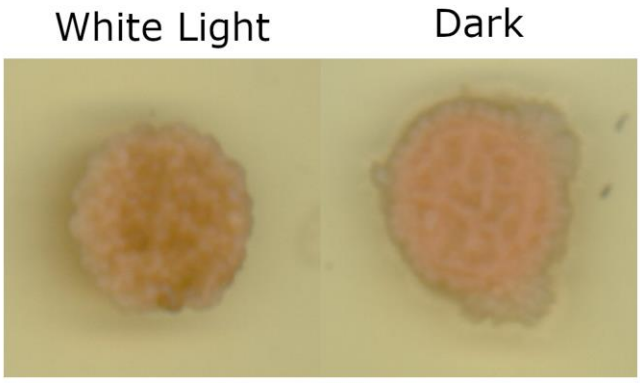


Figure 2.6 Comparison of *Zymoseptoria tritici* cultures grown in day:night cycles and constant dark on yeast-sucrose agar (YSA) plates. Drops (2 μL) containing approximately 107 cells were inoculated on to YSA plates and grown under white light or dark.

2.3 Discussion

The RNAseq analyses show clearly that *Z. tritici* can sense and respond to light. The high numbers of differentially expressed genes between the comparisons indicate that *Z. tritici* alters its transcriptome in the dark compared to when it is growing in the light and between different wavelengths of light. While this is not surprising, as many other fungi have been shown to sense and respond to light, this is the first confirmation that the hemibiotrophic wheat pathogen *Z. tritici* also uses light to trigger large differences in gene transcription, which presumably has a

correspondingly large effect on metabolic processes (Ballario et al. 1996; Lee et al. 2006; Fanelli et al. 2012b; Canessa et al. 2013; Fuller et al. 2013).

Approximately half of the differentially expressed genes (48-63% depending on the comparison) do not have any functional annotations so this is the first time they have been implicated in possible responses to light or dark. The proportions of unannotated genes among those that were differentially expressed in the light and dark treatments were moderately higher than their representations in the genome in general. This could be in part because no other analyses of differential expression in response to light have been performed on *Z. tritici*, but also could indicate that this fungus has different light responses than those that have been seen in other species. Many prior analyses of light responses in fungi have been with the model fungus *N. crassa* in the class Sordariomycetes. Light responses of fungi in the class Dothideomycetes to which *Z. tritici* belongs are less well understood, and it is possible that this species may use different genes or have different responses compared to other fungi. The differentially expressed genes with no functional annotations could indicate that novel pathways are involved in the responses of Dothideomycetes to light. This hypothesis could be tested in the future by knocking out those genes to identify any altered phenotypes in response to different light conditions.

Light affected expression of genes on all 21 chromosomes in the *Z. tritici* genome, including all eight dispensable (or accessory) chromosomes, indicating that light most likely has a significant effect on growth and development of this fungus. The percentages of genes that were differentially expressed in any of the comparisons agrees with what was found previously in *N. crassa*, indicating that while the response was large, this is likely correct (Wu et al. 2014). One interesting result was that genes with annotations relating to oxidative stress, metabolism and transportation were upregulated primarily in the light, while the fungus would be growing on its host during the day, while genes with annotations relating to growth and general cell maintenance were more common primarily in the dark, and therefore would be expressed at night. Further experiments are needed to test whether these *in vitro* experiments reflect gene expression on the host. Unlike the pattern seen in *Z. tritici*, in *Trichoderma atroviride*, transport-related genes were repressed in the wild type when grown under constant light and were expressed in a *wc1* knockout mutant (Cetz-Chel et al. 2016). However, our findings on cellular metabolism are similar to what was found in *N. crassa* previously (Chen et al. 2009; Wu et al. 2014).

The upregulation of genes related to oxidative stress under light indicates another common way that fungi can respond to radiation-induced stresses. Oxidoreductases are involved in reducing free radicals generated by UV radiation so their increased expression in response to light would be adaptive for the fungus. Light has been shown to increase a response to oxidative stresses in other organisms (Canessa et al. 2013; Fuller et al. 2013; Wu et al. 2014; Cetz-Chel et al. 2016) and seems to induce similar responses in *Z. tritici*.

Expression patterns for many genes were as expected based on their predicted biology. For example, cryptochromes are involved in sensing and responding to blue light in other organisms (Bluhm and Dunkle 2008; Castrillo et al. 2013). The primary cryptochrome gene in *Z. tritici*, *Cry*, is upregulated very specifically in white and blue light as would be expected from its biological function. Photolyases repair DNA damage caused by exposure to light, particularly UV (Sancar 1994). One of the two putative *Z. tritici* photolyase genes analyzed was strongly upregulated in response to blue and white light, which would be the closest to UV, while the other was upregulated at a low level. The main photolyase gene, *Phr*, was not differentially expressed in response to light so may have a different function or could be expressed constitutively to protect against DNA damage in general.

In contrast, many genes with light-related biological functions in other organisms were not differentially expressed in the *Z. tritici* experiments. This included light-sensing genes such as *wc1* and the phytochrome genes (*Phy-1/2*). While they may not be differentially expressed under long exposure to light, such as that used here, it is possible that other results would be obtained during transitory periods of light, such as during dawn or dusk, which affects *wc-1* in *N. crassa* (Wu et al. 2014). Many of these genes may be regulated in a similar fashion, with higher differential expression when light conditions are changing. Another possibility is that the major light-sensing proteins are expressed constitutively so that they are available to detect changes when they occur. Additional experiments covering diurnal variations are required to answer these questions.

Proteins likely to be involved in fungal virulence showed some interesting expression patterns. The most obvious of these were the increased transcription of LysM domain-containing genes in response to light. These code for effector proteins that can minimize wheat host defenses in response to *Z. tritici* during the initial invasion phase of infection by binding chitin, and protecting the cell wall from hydrolysis (Marshall et al. 2011). *Mg1LysM* was upregulated by any light, while *Mg3LysM* was upregulated primarily by red light. *Mg3LysM* is the effector primarily

responsible for blocking the wheat host from activating defenses (Marshall et al. 2011), so it is interesting that it would be upregulated in red light, rather than be expressed constitutively. Similarly, while *Mg1LysM* is not actively responsible for blocking a defense response, it is not clear why it would be expressed in the light rather than constitutively unless it has another role related to growth or pathogenicity.

Some other genes that could be linked to pathogenicity, including proteases and SSPs, also showed differential expression in response to light. Very few of the differentially expressed SSPs had any functional annotations other than being flagged as such by Palma-Guerrero et al. (Palma-Guerrero et al. 2017). One of those with an annotation that was downregulated in response to light, ZtritIPO323_04g10536, had a chitin-binding peritrophin-A domain. These domains are found primarily in insects (Huerta-Cepas et al. 2016), and most of the non-orthologous group annotations were from *Drosophila* species. Preventing the plant from sensing chitin is critical for avoiding PAMP-triggered immunity and other responses against fungal invaders, and is the major effect of the LysM effector proteins secreted by *Z. tritici* during infection. Curiously, the expression patterns of ZtritIPO323_04g10536 were opposite those of the LysM genes, which were induced by light. This could be an indication that this is another chitin-binding effector that works opposite to the LysM effectors.

The proteases were fairly evenly divided between being upregulated by light, downregulated by light, or not differentially expressed. Expression of proteases is consistent with the conclusion of Goodwin et al. (Goodwin et al. 2011) that the genome of *Z. tritici* was more similar to those of endophytes rather than other pathogens and that pathogenicity might involve catalysis of proteins rather than carbohydrates during the early stages of infection. However, why most of those genes would have higher expression in response to light is not known. In contrast to most proteases, the one with the highest differential expression, aspartic protease *MgAsp2*, is part of a family of genes that is potentially involved in pathogenicity in *B. cinerea* (ten Have et al. 2004), yet had much higher expression in dark than in light.

Pigmentation is a critical component of photoprotection, or is controlled by circadian rhythms to produce pigments during the day (De Fabo et al. 1976; Avalos and Estrada 2010; Bluhm et al. 2010; Kim et al. 2011a; Canessa et al. 2013; Fuller et al. 2013). Despite this tendency, the pigment-related genes in *Z. tritici* varied in whether they were up or down regulated in the light. The three MAPK-encoding genes provide a good example. All three are required for melanization

(Cousin et al. 2006; Mehrabi et al. 2006b, a), yet each has a different expression pattern. *MgFus3* was not differentially expressed, *MgHog1* was downregulated by light, and *MgSlt2* was upregulated by white and blue light (Figure 2.3B). These genes are also required for full virulence, via different parts of the infection cycle (Cousin et al. 2006; Mehrabi et al. 2006b, a). Another gene that is known to be directly involved in the synthesis of melanin, the polyketide synthase PKS1 (Lendenmann et al. 2014), was downregulated in white light only. It was not differentially expressed in any other comparison, indicating that it may not be photoregulated or is regulated by a color of light outside of blue and red. We found that melanization of *Z. tritici* hyphae occurs independent of light conditions, and this further supports the hypothesis that melanization is not regulated by light in this fungus.

Other genes in the secondary metabolism pathways also are differentially expressed and tend to be upregulated in the light. Nearly half of them were upregulated in the light, and the remaining half were divided between being downregulated and not differentially expressed (Figure 2.3C). This correlates with the findings from the enriched GO terms, where metabolism and secondary metabolism GO classes were expressed more in the light than in the dark (Figure 2.5).

There are many other classes of genes that have been linked to pathogenicity in other species of fungal pathogens, including the PCWDEs, peroxidases, and lipases. The effect of light on these genes varied within classes. There were some general trends, such as nearly half of the PCWDEs were downregulated in light, but due to a lack of detailed annotations, more granular regulation trends are difficult to ascertain. A better understanding of how these genes are expressed in the host under various light conditions is essential for a complete picture of gene expression during infection.

This experiment provided a single snapshot of gene expression under different light conditions, rather than analyzing changes over time or during the transitions from dark to light and vice versa. As such, some genes that may be differentially expressed only during a short period after light exposure would not be found. Previous RNAseq experiments in *N. crassa* show that there are genes that are differentially expressed, both up and down regulated, during the 15 to 240 minutes after initial exposure to light that then return to basal dark expression (Wu et al. 2014). It is highly likely that additional *Z. tritici* genes would have been differentially expressed if samples had been collected during the first few minutes or hours after the transitions to light or dark. For instance, *white collar 1* in *N. crassa* was highly differentially expressed during the first 15 minutes

following exposure to white light, but returned to dark levels of transcription after an hour (Wu et al. 2014).

It was not possible to conclude which color of light stimulates the greatest response from *Z. tritici*. The expression patterns under red and blue light were very similar, with only a small percentage of genes differing between these two light-versus-dark comparisons. The white light versus dark comparison had the highest number of differentially expressed genes among the three light treatments, but since blue and red are both components of white light this does not help to identify which color can be sensed most efficiently. The genome sequence of *Z. tritici* (Goodwin et al. 2011) appears to have more genes for sensing and responding to blue light, including the WCC, VIVID, cryptochrome, and photolyase genes compared to only a single phytochrome gene for red. Biologically it may make sense for the fungus to have a heightened ability to sense and respond to blue light, as this would most likely co-occur with damaging UV, while red light alone would be present in nature mostly when the light is changing near sunrise or sunset and the amount of UV would be lower.

2.4 Conclusions

These limited initial experiments on gene expression show that *Z. tritici* can sense and respond to light, with profound effects on growth, development and metabolism. The large number of functionally unannotated genes involved in light responses indicates a huge gap in our knowledge that must be filled before we can fully understand the effects of light on this fungus. We still know nothing about the minimum intensity of light that can trigger a response, how long light must be present before a response is initiated, or whether the fungus can specifically sense UV. Differential expression of various genes potentially or proven to be involved with infection indicates that light could be important for pathogenicity, and other effectors may show a similar pattern of transcription. A better understanding of the effects of light on pathogenicity and other biological processes of this fungus could provide the basis for development of improved disease management strategies in the future.

2.5 Methods

2.5.1 Fungal growth and light treatments

The *Zymoseptoria tritici* isolate IPO323 was used for these analyses, as it has been sequenced completely, grows readily in culture and, as an excellent reference genome, has been the subject of much prior research (Goodwin et al. 2011). This isolate was grown on sealed yeast-sucrose agar plates (YSA; 10.0 g/L yeast extract, 10.0 g/L sucrose, 15.0 g/L BactoAgar) under filters to control the wavelengths of light received by each culture. The treatments were white light (no filter), blue (400–530 nm) and red (600–700 nm) light using colored acrylic glass filters (American Acrylics, Skokie, IL) (Kim et al. 2011a). Full dark was achieved by wrapping the plates in aluminum foil. Light for the cultures was provided by 32W fluorescent bulbs. Each condition had three biological replicates consisting of one 9-cm Petri dish each. The three light conditions had 16:8 day:night cycles. The cultures were initiated with plugs taken from the margins of an actively growing mycelium and grown for a week at 23^{o}C.

2.5.2 RNA extraction and sequencing

Fungal tissue was quickly scraped from the agar, frozen immediately in liquid nitrogen for grinding, and total RNA from the cultures was extracted using a QIAGEN RNeasy Plant Mini Kit (Catalog Number 74903), following the manufacturer's recommendations. The RNA was then sent to the Purdue Genomics Core Facility (West Lafayette, IN) to be processed and sequenced on an Illumina HiSeq 2500. The data were downloaded to the Halstead Computing Cluster of the Purdue Rosen Center for Advanced Computing for analysis.

2.5.3 RNAseq and statistical analyses

Trimmomatic (version 0.36) was used to remove leading and trailing bases with a phred score lower than 30, and reads that were shorter than 40 base pairs long after base removal (Bolger et al. 2014). HISAT2 (version 2.0.5) was used to map the remaining reads to the *Z. tritici* reference genome (Langmead and Salzberg 2012; Sirén et al. 2014; Kim et al. 2015a). Samtools (version 1.4) was used to sort the mapped reads, and HTSeq-count (version 0.7.0) was used with the King

Rothamsted reference annotation to obtain a counts table for genes (King; Li et al. 2009; Anders et al. 2015).

The creation of the reads library was done by using the paired-end reads from each of three biological replicates for each of the four light conditions. The table of gene read counts was exported to R (version 3.4.0). The gene read counts were cleaned by removing any genes that had a rowsum of fewer than 100 reads totaled over all samples, and any genes where any one replicate had fewer than 10 reads, in that order. This conservative approach was used to limit the analysis to genes with reliable data for all replicates. DESeq2 (version 1.15.51) was used to calculate differential gene expression and to obtain adjusted p values and log2 fold changes.

A custom Perl script was used to pull gene sequences from the reference annotation and the gene sequences were analyzed by eggnog-mapper (version 4.5.1) and InterProScan (version 5.24-63.0) (Jones et al. 2014; Huerta-Cepas et al. 2016). Another custom Perl script was used to collect each GO term assigned to each gene in a gene-to-GO table, which was fed in to TopGO (version 2.28.0) (Alexa and Rahnenfuhrer 2016). These Perl scripts are available on request. Lists of differentially expressed genes from DESeq2 were used to determine overexpressed GO terms found in each comparison.

GOOSE (GO Online SQL Environment) was used to query the Gene Ontology (GO) database to generate levels (Alterovitz et al. 2007; Carbon et al. 2009). The maximum tree depth was used to organize the enriched GO terms for visual analysis. CateGOrizer was used to map the biological processes of each enriched GO term to a parent term in the standard GO_slim (Hu et al. 2008). *ggplot2* was used to generate pie charts from the CateGOrizer data (Wickham 2009).

CHAPTER 3. THE ROLE OF A VIVID HOMOLOG IN GROWTH AND PATHOGENICITY OF *ZYMOSEPTORIA TRITICI*

3.1 Introduction

The ascomycete *Zymoseptoria tritici* (syns: *Mycosphaerella graminicola, Septoria tritici*) is the causal agent of Septoria tritici blotch (STB), a very important fungal disease of wheat (*Triticum spp.*). This disease is not only found around the world wherever wheat is grown, but also can cause major yield losses, reaching up to 50% when left uncontrolled (Fones and Gurr 2015). The disease cycle of *Z. tritici* begins when an ascospore or conidium lands on a leaf and germinates. The germ tube invades the stomata, and the hyphae grow through the apoplast of the leaf without causing any symptoms. After this latent period, necrotic lesions form on the leaf, and the asexual pycnidia form within the stomatal cavities and release conidia to continue the cycle. Pseudothecia from the sexual cycle form on the infected leaves, and provide the primary overwintering structures and initial inoculum for the next season (Kema et al. 1996; Duncan and Howard 2000; Ponomarenko et al. 2011).

Control of this pathogen is primarily based around fungicide treatments and the breeding of resistant wheat cultivars (Fones and Gurr 2015; Figueroa et al. 2018). *Z. tritici* is the largest target of fungicide use in wheat (Torriani et al. 2015). These methods are far from perfect. *Z. tritici* has a large amount of genetic diversity in the field due to frequent sexual recombination, with a concomitant ability to develop resistance to fungicides and to overcome host resistance (Fraaije et al. 2005; Brown et al. 2015). There are three primary classes of fungicides used to control STB: the quinone outside inhibitors (QoI), the demethylation inhibitors (DMI), and the succinate dehydrogenase inhibitors (SDHI). There is widespread resistance to the QoI fungicides, resistance is beginning to form against the DMI fungicides, and there is a high risk of resistance forming to the SDHI class of compounds (Fraaije et al. 2005; Wieczorek et al. 2015; Hayes et al. 2016; Cheval et al. 2017; Huf et al. 2018; Rehfus et al. 2018).

Light is an important environmental factor for all fungi, including those in the class Dothideomycetes such as *Z. tritici.* Light can cause great amounts of stress in fungal tissues, damaging the DNA and forming reactive oxygen species (ROS) (Fuller et al. 2015). As such, fungi use light as an environmental cue for many different processes. These processes are wide ranging, including general growth patterns, pigmentation, reproduction, secondary metabolism, and can

even include pathogenicity and virulence (Thorold 1940; Linden et al. 1997; Daub and Ehrenshaft 2000; Bahn et al. 2006; Hatakeyama et al. 2007; Bluhm et al. 2010; Wang et al. 2010; Kim et al. 2011c; Schmidt-Heydt et al. 2011; Kim et al. 2011b; Canessa et al. 2013; Röhrig et al. 2013; García-Martínez et al. 2015; Schumacher 2017; Yu and Fischer 2018; Tang et al. 2020).

The pathways ascomycetes use to sense blue light are highly conserved throughout the Ascomycota (Corrochano 2019). The primary protein used to sense blue light is known as White Collar 1 (WC1) (Ballario et al. 1996; Lee et al. 2003; Yu and Fischer 2018). This protein forms a complex with White Collar 2 (WC2) to form the White Collar Complex (WCC) (Talora et al. 1999; Yu and Fischer 2018). The WCC acts as a transcription factor, to induce and repress expression of various blue-light-regulated genes when light is present. This light response is mediated by a protein known as VIVID (VVD), which senses light, then binds to and represses the action of the WCC (Schwerdtfeger and Linden 2003; Chen et al. 2010; Gin et al. 2013). This repression of light responses is known as photoadaptation and leads to fungi returning gene expression to a more basal state and giving them the ability to differentiate between different light intensities (Schwerdtfeger and Linden 2003). Both WC1 and VVD utilize a flavin-based chromophore for sensing light (Froehlich et al. 2002; He et al. 2002; Schwerdtfeger and Linden 2003; Yu and Fischer 2018).

Light responses in *Z. tritici* are not well characterized. The reference genome of *Z. tritici* (Goodwin et al. 2011) contains all of the major light-sensing genes found in Ascomycota. It has been shown that *Z. tritici* does sense and respond to light, showing differences when grown in constant darkness versus in a day:night cycle (Tiley et al. 2019). *Z. tritici* also shows large differences in gene expression in response to light (McCorison and Goodwin 2020). The precise effects that light-sensing proteins have in the growth and development of *Z. tritici* have not yet been characterized. In other fungi, deletion mutants of VVD homologs cause alterations in reproduction, changes in pigment production, and shows that it plays a minor role in controlling the circadian rhythm (Shrode et al. 2001; Hunt et al. 2007; Carreras-Villaseñor et al. 2012; Castrillo and Avalos 2014; Zhang et al. 2017b; Schmoll 2018). The goal of this research is to test the hypothesis that the deletion of *ZtVVD* will affect the production of pigments, and that it will affect the survivability of *Z. tritici* under various stress conditions. These stress conditions include those generated by light, such as high UV conditions, oxidative stress, and other stresses such as nutrient stress and osmotic stress.

3.2 Materials & Methods

3.2.1 Fungal Strains and Culture Conditions

The *Z. tritici* reference strain IPO323 was used as the wild type and as the basis of the gene disruptions done in this study. The initial culture conditions were on potato dextrose agar (PDA) (1.5% agar, Difco) plates at 19^{o}C in darkness. Strains were stored suspended in 25% glycerol at -80^{o}C for medium-term storage, and after being allowed to grow onto sterilized Whatman filter paper, then desiccated for 24 hours in a lyophilizer, cut into strips and placed in sterile vials at -80^{o}C for long-term storage. Phenotyping culture conditions were done on PDA amended with the specified compound unless reported otherwise, such as YSA (yeast sucrose agar, 10 g of yeast extract, 10 g of sucrose, 1.5% agar per liter) or water agar (1.5% agar). The studied light conditions were 16:8 hours day:night under fluorescent light; full darkness was achieved by wrapping the plates in aluminum foil. Culture temperatures were room temperature (approximately 22^{o}C). All plates were sealed via airtight bags, or with parafilm (Bemis).

3.2.2 Protein Alignment and Analysis

The protein sequences of known functional VIVID proteins from *Neurospora crassa* (GenBank accession number AAK08514.1), *Cordyceps militaris* (GenBank accession number AMN38423.1), and *Fusarium fujikuroi* (GenBank accession number XP_023432193.1) were downloaded from the NCBI GenBank database and aligned with that of the *Z. tritici* VIVID protein (accession number XP_003849613.1) using the MUSCLE aligner in MEGA-X (version 10.1.7).

3.2.3 Nucleic Acid Manipulation

IPO323 genomic DNA was extracted using a Qiagen Plant DNeasy kit (cat #: 69104). A double-jointed PCR method was used to generate the replacement construct (Yu et al. 2004). The primer combinations ZtVVD-A/ZtVVD-B-HPH and ZtVVD-C-HPH/ZtVVD-D were used to generate the flanking regions from IPO323 genomic DNA (Table 3.1). These primers were designed using the reference genome sequenced by the U.S. Department of Energy Joint Genome Institute (Goodwin et al. 2011). Primers HPH-F/HPH-R were used to generate the hygromycin phosphotransferase (*hph*) segment of DNA from the plasmid pBHT2 (Table 3.1) (Mullins et al.

2001). These DNA fragments were purified using gel electrophoresis and extraction using a Qiagen QIAquick Gel Extraction kit (cat #: 28115) and then combined without primers in a 1:3:1 molar ratio (Flank AB:*hph*:Flank CD) and joined using a double-jointed PCR method (Yu et al. 2004). The reaction was used directly in a third PCR using primers ZtVVD-Nest-F/ZtVVD-Nest-R, which contain recognition sites for the restriction enzymes XbaI and HindIII, respectively (Table 3.1). The product was purified using an isopropanol-sodium acetate method and cloned into pBHT2 using the aforementioned restriction enzymes (XbaI: New England Biolabs cat #: R0145, HindIII: New England Biolabs cat #: R3104) and T4 DNA ligase (New England Biolabs cat #: M0202). This plasmid, designated pZVVD was cloned into competent TOP10 *E. coli* cells (Thermo Fisher cat #: C404010) and extracted at higher concentration using a Qiagen QIAprep Spin Miniprep kit (cat #: 27104). Extracted pZVVD was transformed into competent cells of *Agrobacterium tumefaciens* strain Agl1 using standard electroporation methods (Weigel and Glazebrook 2006).

Table 3.1 Primers used in transformation and confirmation of ZtVVD mutants in the wheat pathogen *Zymoseptoria tritici*.

Primer Name	Primer Sequence (5' → 3') [a]
HPH-F	GTCCAGGCGGTGAGCACAAAA
HPH-R	TTCCCGGTCGGCATCTACTCTATTC
ZtVVD-A	CCTTGATGGGAGGGAGAGGA
ZtVVD-B-HPH	TTTTGTGCTCACCGCCTGGAC TGCAAGAGGGATAGCTGCAT
ZtVVD-C-HPH	GAATAGAGTAGATGCCGACCGGGAA GTCAGCGGCCTCGTACTATC
ZtVVD-D	GATTGGTCGGAGGGCTGAAA
ZtVVD-Nest-F	TCGTTACATCATCCCTtctagaGACTTGAAA
ZtVVD-Nest-R	CTGCTGAGTGGTAGATaagcttATACTGTCG
ZtVVD-Ver-F	CCTCTCCCTCCCATCAAGGA
ZtVVD-Ver-R	TGCGCATAGGTGCATACAGA
ZtVVD-Neg-F	GTTCCATCGCACGGGTATCA
ZtVVD-Neg-R	ATACTGGTAGGTGTCCGCAT

[a] Underlined bases are the overlapping hph sequence, and lowercase letters are restriction enzyme recognition sites.

3.2.4 Agrobacterium-Mediated Transformation and Verification

Knockout mutants were generated as in Choi and Goodwin (2011) with modifications using *A. tumefaciens* strain containing pZVVD. The initial cultures were grown from frozen IPO323 glycerol stock (25% glycerol) on YSA plates for 4 days at 19ºC. No filter papers were used; the cultures were transferred directly from one plate to the other by pressing the face of one agar culture to the face of the fresh medium.

The hygromycin-resistant colonies were transferred to selective media, and subcultured to PDA containing only 100 U of hygromycin. The DNA was extracted and used for a double PCR for verifying the insert presence using the primers in Table 1. The ZtVVD-Neg pair amplifies the wildtype ZtVVD gene, while ZtVVD-Ver amplifies over the insert (Figure 3.1).

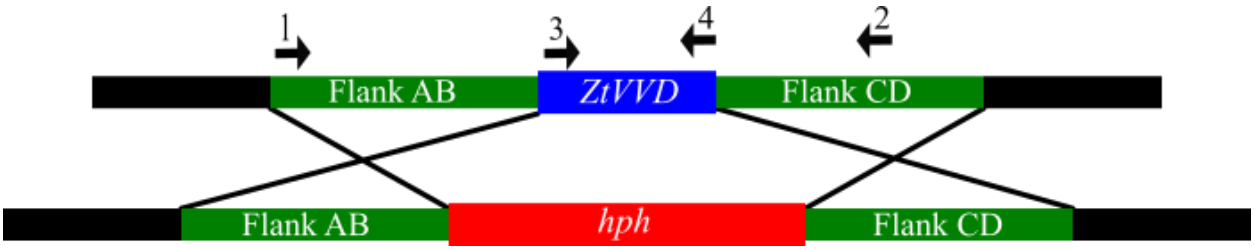


Figure 3.1 Diagram of replacement construct and primer locations for making a knockout mutant of the ZtVVD gene in the wheat pathogen *Zymoseptoria tritici*. Not to scale. 1 and 2 indicate the ZtVVD-Ver primers, which amplify over the inserted region, and 3 and 4 indicate the ZtVVD-Neg primers, which amplify the intact ZtVVD gene.

3.2.5 Agar Plate Phenotyping

Cells from a 4-day-old PDA plate were resuspended in sterile water and adjusted to 10^7 cells/mL, 10^5 cells/mL, and 10^3 cells/mL. A 2-uL aliquot of each strain and concentration was inoculated onto each phenotyping plate, then was left to dry, bagged and placed into the specified conditions. The plates were allowed to grow for 10 days before being scanned using an Epson Photo 6800 flatbed scanner at 1200 dots per inch.

3.2.6 Germination Tests

Germination tests were performed by inoculating water agar with approximately 10 spores of *Z. tritici.* The plates were sealed, and incubated at 19^{o}C for 30 days, then analyzed on a Leica dissecting microscope. Images were taken using the camera on a Google Pixel 4 phone held in place with a 3-D-printed holder.

3.2.7 Pathogenicity and Virulence Studies

Seeds of the highly susceptible wheat cultivar Taichung 29 were grown to 14 days old under standard 16:8 day:night cycles under fluorescent grow lights. The three inoculums were made by scraping a 4-day old plate of each strain, and adjusting a suspension in water to 10^7 cells/mL with a hemocytometer. Tween 20 was added to 0.1% just before inoculation with cotton swabs. The inoculated plants were placed into bags to encourage high humidity and left for 72 hours. The bags were removed, and the plants were left to grow for an additional 11 days before the disease incidence was quantified at 21 days post inoculation (dpi) and again at 28 dpi by scanning in the first two detached leaves on an Epson Photo 6800 flatbed scanner at 1200 dots per inch.

3.2.8 Preparation of Cytology Samples

Fourteen-day-old seedlings of Taichung 29 were inoculated and treated as described in the previous section. At 10 dpi, the lowest leaf was detached, cut into 2-cm pieces, and boiled in 1:2 lactophenol:ethanol (Cat # Sigma Aldritch R03465) for 15 minutes, then transferred to saturated 5:2 wt:vol chloral hydrate:water (Cat # Sigma Aldritch C8383) for approximately 16 hours for clearing (Mehrabi et al. 2006b, a). The destained leaf sections were then analyzed under a microscope with a 10x objective (Leica Insert model here), and images were taken with a Google Pixel 4 held with a 3-D printed holder. The stained leaf sections were then stored in 87% glycerol.

3.3 Results

3.3.1 Homology to the *N. crassa* gene VIVID

The *Z. tritici* VIVID protein sequence (accession number XP_003849613.1) was compared to sequences of known functional VIVID proteins from *N. crassa* (accession number AAK08514.1), *Cordyceps militaris* (accession number AMN38423.1), and *Fusarium fujikuroi* (accession number XP_023432193.1). ZtVVD does not have high identity with the other VIVID proteins (*N. crassa* VIVID: 34.6% identity; *F. fujikuroi*: 32.3%;, *C. militaris*: 29.3%), however it is typical for a protein with similar function from different taxonomic classes (Figure 3.2).

The flavin chromophore of the VIVID protein binds via conserved amino acids. Seven of amino acids in the flavin binding site identified in *N. crassa* VIVID are present in ZtVVD (Figure 3.2). The remaining 3 amino acid residues that are not identical are similar so may function identically, such as both being positively charged amino acids or both small nonpolar amino acids. The conserved photoactive cystine residue from *N. crassa* also is present in ZtVVD (Figure 3.2) (Schwerdtfeger and Linden 2003). These similarities indicate that ZtVVD almost certainly can successfully bind the flavin chromophore, and therefore can act as a photosensor.

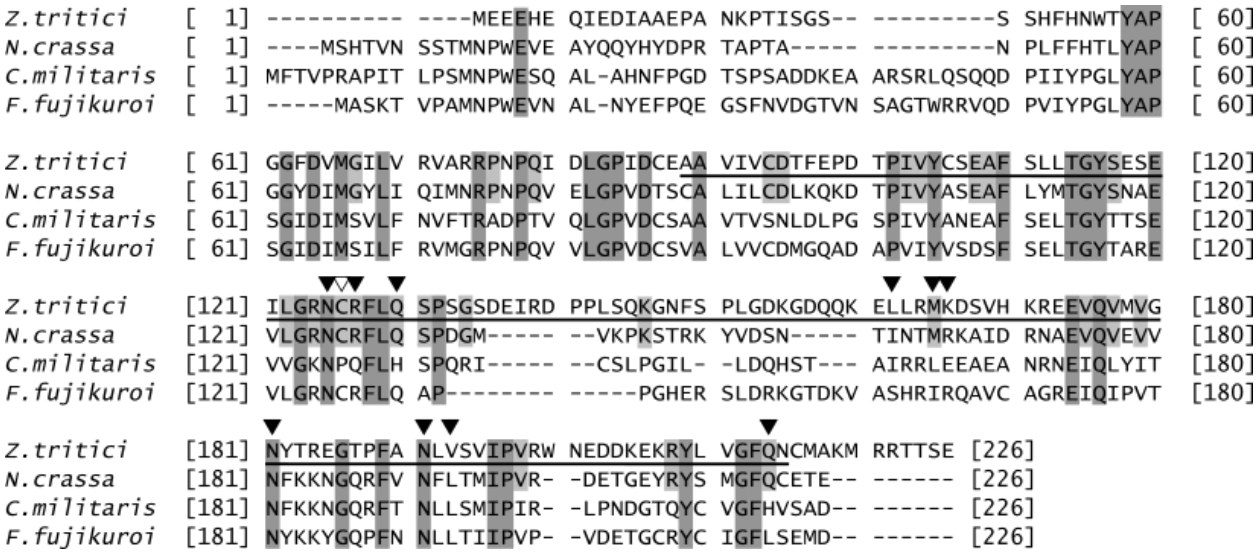


Figure 3.2 Alignment of ZtVVD from the wheat pathogen *Zymoseptoria tritici* with VIVID from *Neurospora crassa* (AAK08514), *Cordyceps militaris* (AMN38423), and *Fusarium fujikuroi* (XP_023432193) (Schwerdtfeger and Linden 2003; Castrillo and Avalos 2014; Zhang et al. 2020). The black arrows indicate amino acid residues in the flavin-binding site in *N. crassa* VIVID and the open arrow indicates a photoactive cystine (Schwerdtfeger and Linden 2003). The dark-shaded residues are identical between all four VIVID sequences, and the lighter ones are identical between the *Z. tritici* and *N. crassa* VIVID sequences. The underlined section indicates the PAS-9 (Per-Arnt-Sim-9) domain from *Z. tritici* (acc. number F9XK26) according to the Pfam database, which is the primary binding site of VVD, to both the chromophore and to other proteins (El-Gebali et al. 2019).

3.3.2 Effect on Growth and Development

Deletion of ZtVVD is not lethal in *Z. tritici.* The growth rate and colony radius of the wild type, knockout and ectopic mutant strains are the same on all media types (Figures 3.3, 3.4).

The gene disruption has minor effects on the general growth of *Z. tritici.* When grown on PDA, ΔZtVVD produces more aerial hyphae than the wild type strain when grown in constant darkness, as indicated by the white, fuzzy appearance of the colonies (Figure 3.4). When grown on YSA, a complete nutrient-rich medium, and in day:night cycles, ΔZtVVD is more melanized and seems to transition to hyphal growth earlier than IPO323 or the ectopic mutant, EctZtVVD (Figure 3.4).

The germination patterns of the ΔZtVVD strain were slightly different compared to IPO323 or the ectopic strain when single-cell growth was examined (Figure 3.5). ΔZtVVD was less clumped and more spread out than the IPO323 or EctZtVVD colonies.

There were differences between growth of the IPO323 strain under the day:night cycles and constant dark conditions (Figure 3.4). As noted previously, IPO323 appears to transition to hyphal growth earlier when grown in constant darkness, as opposed to day:night cycles (Tiley et al. 2019). IPO323 appeared more melanized when grown in the dark on YSA (Figure 3.4).

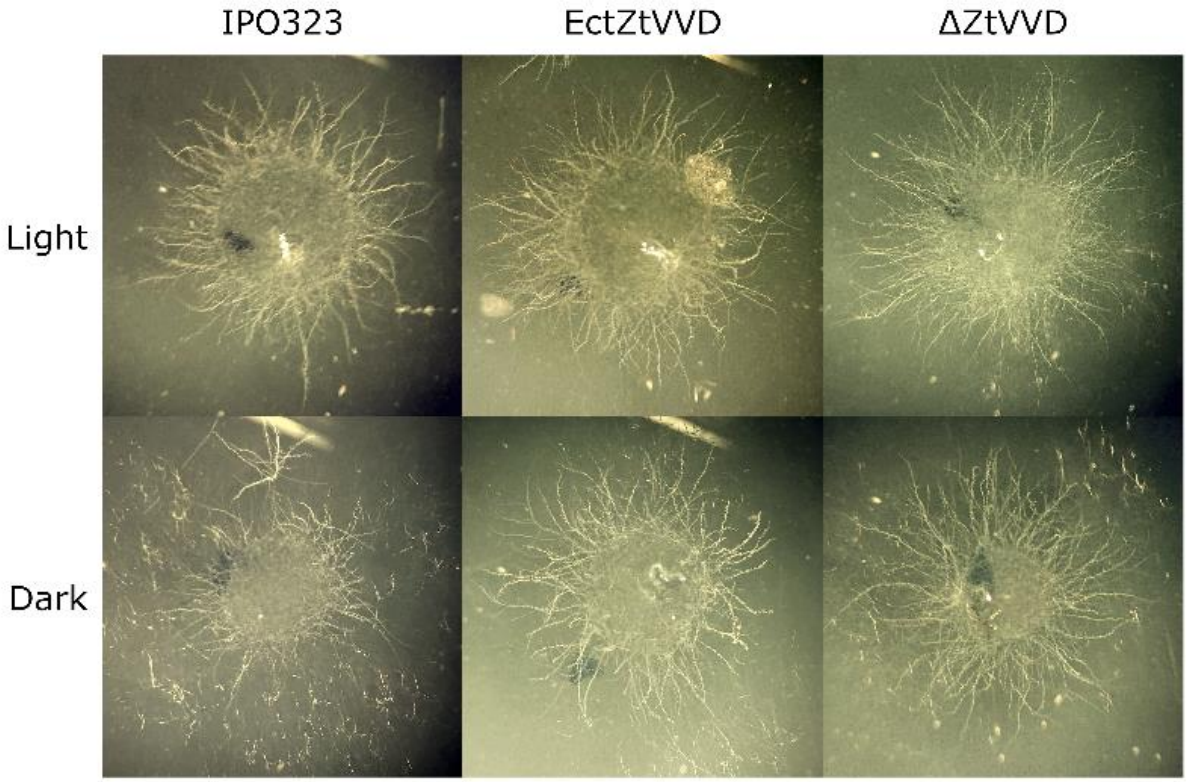


Figure 3.3 Colonies of the three strains of *Zymoseptoria tritici* when grown under nutrient stress. Concentration shown is 10^7 cells/mL but similar results were obtained with other conidial densities.

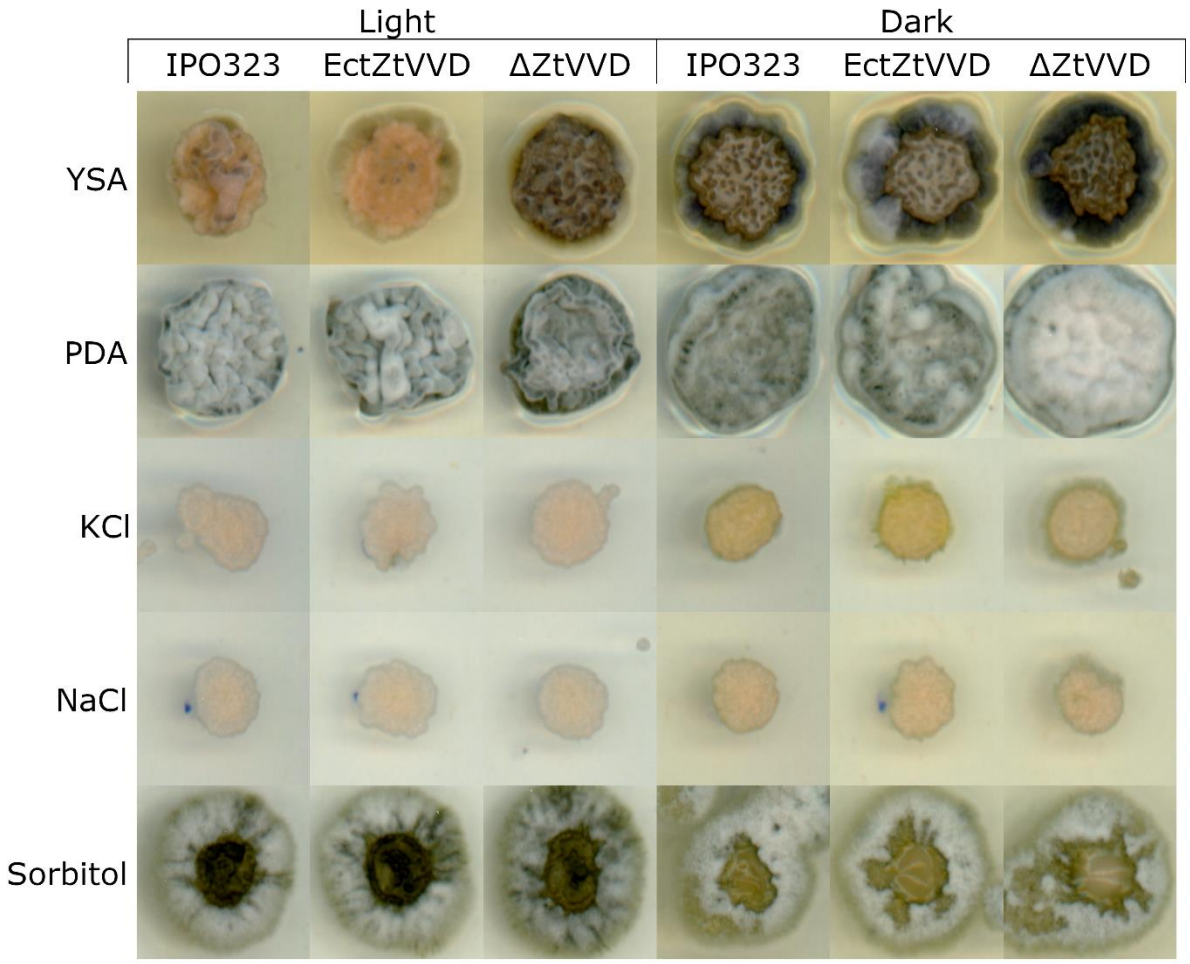


Figure 3.4 The wild type (IPO323), ectopic (Ect) and deletion (Δ) mutant strains of the ZtVVD gene of *Zymoseptoria tritici* grown on the media indicated on the left. Concentration shown is 10^7 cells/mL; other concentrations gave similar results.

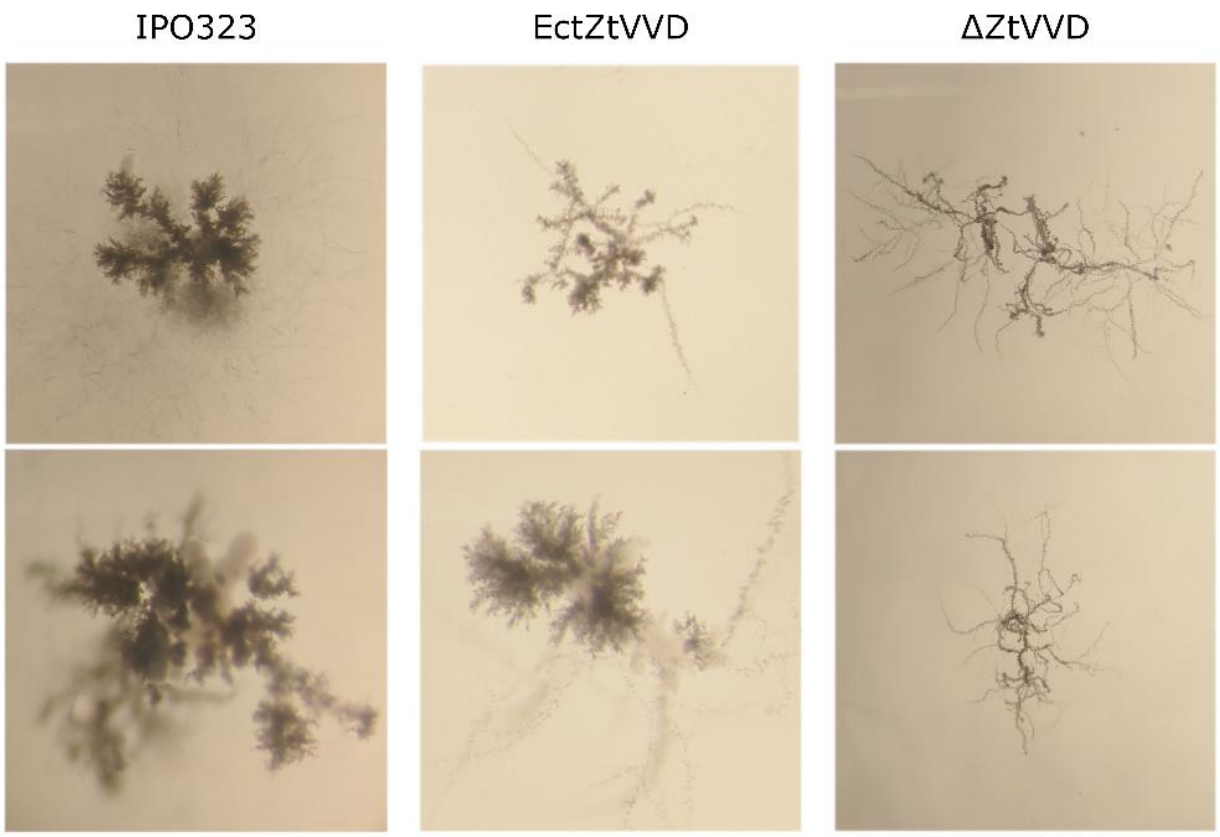


Figure 3.5 The colonies resulting from a single cell of *Zymoseptoria tritici* when grown on water agar for 30 days. IPO323: wild type (untransformed); EctZtVVD: ectopic insertion of the hygromycin gene; ΔZtVVD: knockout mutant of the ZtVVD gene. All colonies were grown under the same conditions.

3.3.3 Response to Stressors

Despite the minor growth phenotypes noted under non-stress conditions, there are no difference between the wild type and ectopic strains versus the ΔZtVVD strain when grown under osmotic stress, on media containing 1 M NaCL, KCl or sorbitol (Figure 3.4). All three osmotic stress conditions have similar growth radii and growth phenotypes.

Between the light and the dark of the osmotic stresses in the wild type, there appears to be a small but consistent difference in the melanization of where it appears that *Z. tritici* when grown in the light under the osmotic stabilization effects of sorbitol (Figure 3.4).

The ΔZtVVD strain did not seem to be hindered in general survival at various temperatures (Figure 3.6). The ΔZtVVD strain did show a consistent greater melanization at 19^{o}C compared to the wild type and the ectopic strains but did not appear to be deficient in survival or growth radius under any temperature conditions. None of the strains grew well at 4 ^{o}C, and all had transitioned fully to growing in hyphal form in 27 ^{o}C.

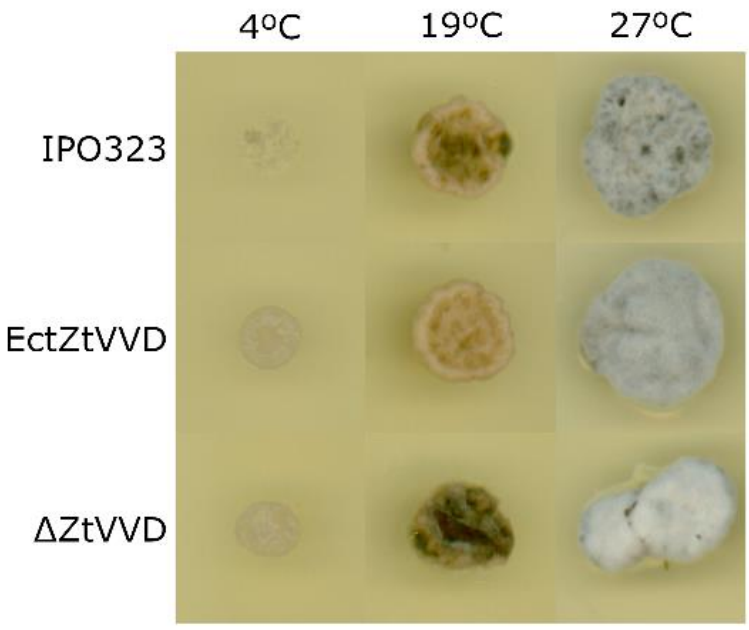


Figure 3.6 The wild type (IPO323), ectopic (Ect) and deletion (Δ) mutant strains of the ZtVVD gene of *Zymoseptoria tritici* when grown at the temperatures shown at the top. Concentration shown is 10^7 cells/mL; other concentrations gave similar results.

3.3.4 Response to Oxidative Stress

The ability of *Z. tritici* to survive oxidative stress appears to be tied to the presence or absence of light under normal conditions when inoculated at low concentrations. The wild type survived under both concentrations of H_2O_2 under day:night conditions but could not survive when in 24-hour darkness (Figure 3.7).

The ΔZtVVD strain, however, survived at 1.0 mM H2O2 in the dark, but not in the light, and survived under both lighting conditions on 0.5 mM H_2O_2 (Figure 3.7). This indicates that ZtVVD reduces the ability of *Z. tritici* to survive oxidative stress. As the function of VIVID proteins is to downregulate the WCC, which has been found to control responses to oxidative stress in other species, this makes sense (Canessa et al. 2013; Fuller et al. 2013).

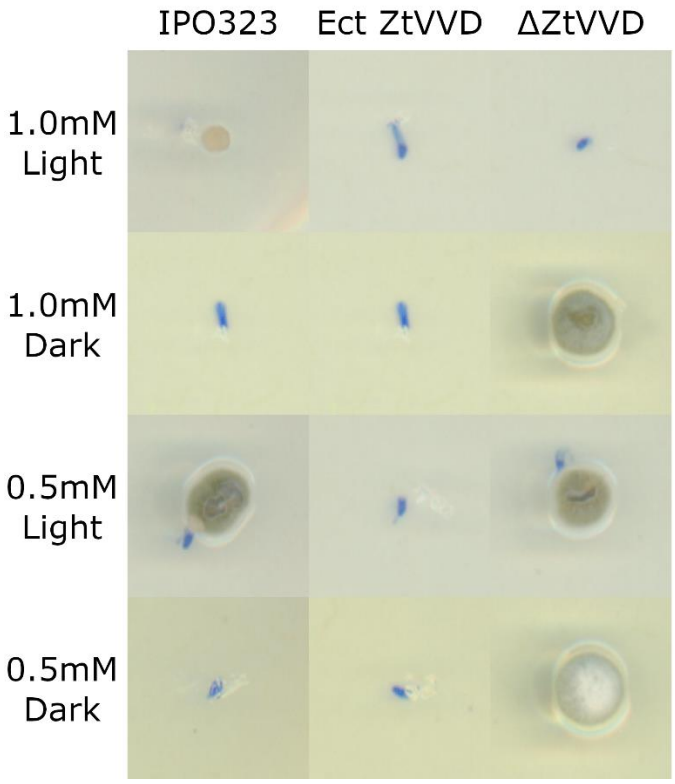


Figure 3.7 The wild type (IPO323), ectopic (Ect) and deletion (Δ) mutant strains of the ZtVVD gene of *Zymoseptoria tritici* grown on potato dextrose agar amended with the concentration of H_2O_2 in the light or dark as listed on the left. Concentration of inoculum shown is 10^3 cells/mL; other concentrations gave similar results.

3.3.5 Survival of UV Radiation

The absence of ZtVVD does not affect the ability of *Z. tritici* to survive UV radiation (Figure 3.8). This is not entirely surprising, as the WCC is the primary inducer of gene expression of genes involved in UV repair and survival. As the VIVID class of proteins down-regulates the function of WCC, it is not likely that the absence of ZtVVD will affect the ability of *Z. tritici* to survive UV radiation.

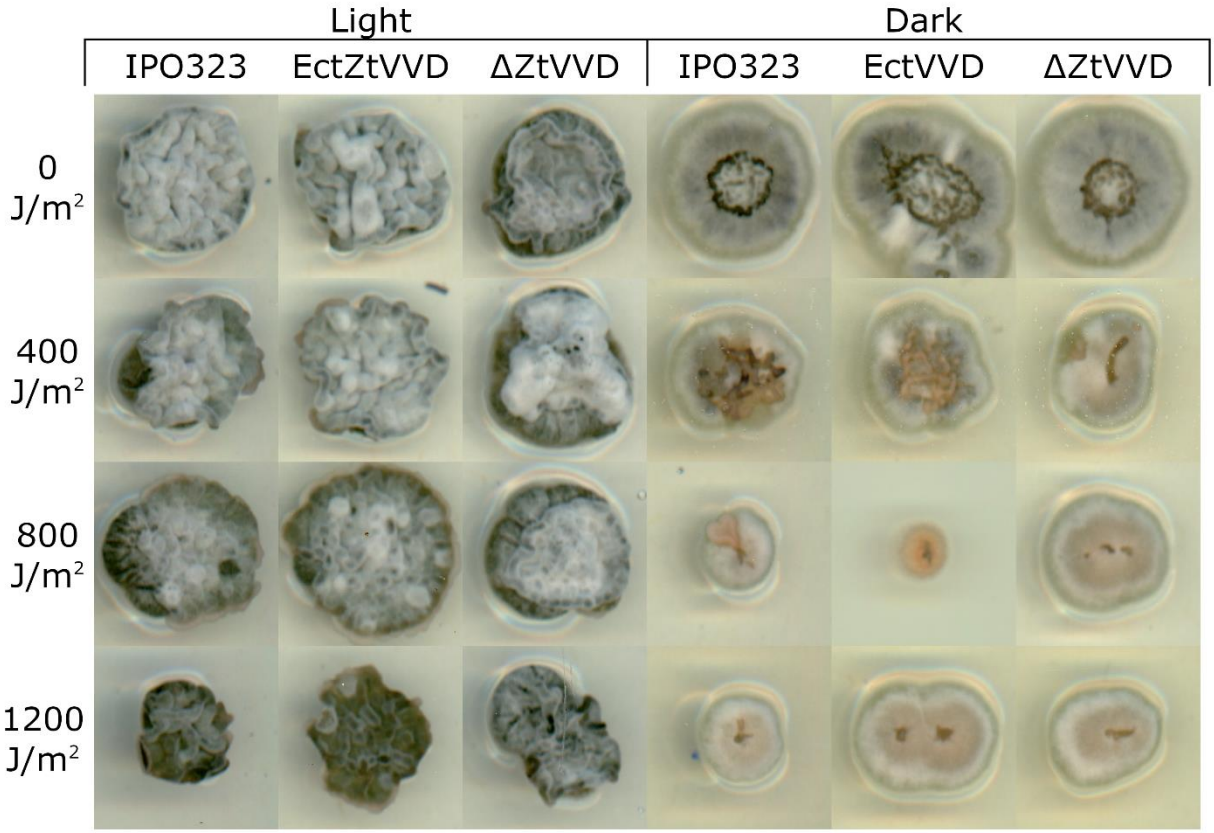


Figure 3.8 The wild type (IPO323), ectopic (Ect) and deletion (Δ) mutant strains of the ZtVVD gene of *Zymoseptoria tritici* after exposure to the amount of UV radiation indicated on the left and subsequently placed in the light conditions indicated at the top. Concentration of inoculum shown is 10^7 cells/mL; other concentrations gave similar results

3.3.6 Effect on Pathogenicity

The ΔZtVVD strain shows no sign of necrosis or pycnidia formation on susceptible plants, while the wild type and ectopic strains both showed typical signs of necrotic lesions and pycnidia on wheat leaves at 21 dpi (Figure 3.9).

Further investigation revealed that the ΔZtVVD strain fails to invade the wheat apoplast. When cleared and stained using aniline blue, the fungal penetration in the wild type and ectopic strains can be seen as blue-stained hyphae between the cells of the apoplast at 10 dpi (Figure 3.10 A-B). In the wheat leaves inoculated with ΔZtVVD, there is no sign of dark blue hyphae between the plant cells (Figure 3.10 C). This indicates that the infection deficiency occurs during initial penetration, and not further into the infection process, for instance a deficiency in the transition from the latent phase to necrotrophic growth.

Figure 3.9 Images of wheat leaves inoculated with the indicated strain of *Z. tritici*, shown 28 dpi.

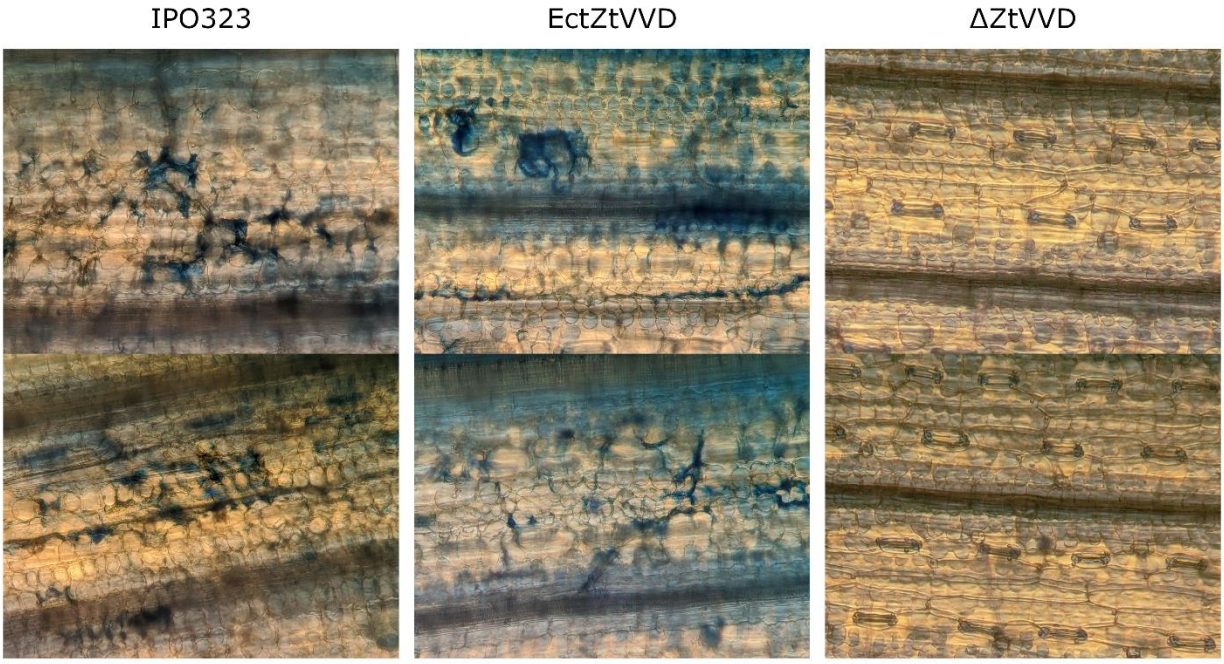


Figure 3.10 Microscopic images showing fungal penetration of the indicated strains of *Zymoseptoria tritici* stained blue with aniline dye. Each image is from a separate leaf.

3.4 Discussion

The photobiology of fungi has recently become a topic of interest. No analyses have been done on the light-sensing proteins in *Z. tritici*. Previous research suggests that this fungus shows a minor change in growth patterns in light on PDA and YSA, and that MVE1 and ZtvelB, homologs of the velA and veB genes respectively from *Aspergillus fumigatus*, have significant effects on growth and melanization (Choi and Goodwin 2011; Tiley et al. 2019). The WC1 protein has been analyzed in other species in the Dothideomycetes (Kihara et al. 2007; Kim et al. 2011a; Pruß et al. 2014; Igbalajobi et al. 2019), but these analyses have not included VIVID and related proteins.

The production of pigments by fungi is often tied to light. These pigments are assumed to protect against the effects of UV radiation, preventing damage to cells and DNA by absorbing excess energy and by detoxifying reactive oxygen species formed by UV. As such, the production of pigments is often induced by light (De Fabo et al. 1976; Harding and Turner 1981; Avalos and Estrada 2010; Bluhm et al. 2010; Hubballi et al. 2010; Canessa et al. 2013; Fuller et al. 2013; Yang and Dong 2014). The light regulation of pigmentation is often mediated via the WCC (Harding and Turner 1981; Kim et al. 2014a; Yang et al. 2016; Krobanan et al. 2019). Similarly, a disruption

in a VVD homolog causes an overproduction of pigments in *N. crassa* and *C. militaris* (Schwerdtfeger and Linden 2001; Zhang et al. 2020). While both species produce carotenoid pigments as opposed to the melanin produced by *Z. tritici*, the phenotype is very similar and supports the findings in *Z. tritici* that more melanin is produced by the VVD gene knockout mutant under certain conditions.

Light is a major stressor for fungi that are attempting to infect host plants, and genes related to sensing light have been shown to be critical in pathogenicity in numerous species across the Ascomycota (Thorold 1940; Kim et al. 2011a, c; Canessa et al. 2013; Adam et al. 2018; Tang et al. 2020). One of these species is of particular interest due to a similarity in infection process and a close relationship with *Z. tritici*. *Cercospora zeae-maydis*, a species of fungus that causes grey leaf spot on corn (*Zea mays*), requires light to form appressoria, and a functional WC1 homolog is required to recognize and invade stomata and cause disease (Kim et al. 2011a). The combination of those functions indicates that light may be required for infection in *C. zeae-maydis*. *Z. tritici* infects wheat via the stomata as well, although *Z. tritici* does not form appressoria within the host leaf.

The lack of penetration by the ΔZtVVD mutant of *Z. tritici* into wheat, and the lack of penetration due to alteration of another light-sensing gene in t *C. zeae-maydis* indicate that casual mechanism of these phenotypes may be related (Kim et al. 2011a). The critical question regarding the lack of infection in the ΔZtVVD mutant is whether the fungus can recognize and invade the stomata, or if the disruption of ZtVVD causes a breakdown of the ability of *Z. tritici* to successfully enter the apoplast of the host plant. This breakdown could be due to an inability to avoid the defenses of the host plant, or *Z. tritici* may be unable to enter the stomata. The ideal way to test these hypotheses would be to observe a recently inoculated plant and examine the surface for signs of stomatal invasion. Previous analyses of the pathogenesis of *Z. tritici* have shown precisely how the fungus penetrates the stomata, providing an excellent basis for understanding the wild type infection mechanisms (Kema et al. 1996; Duncan and Howard 2000).

3.5 Conclusions

VIVID is an important class of proteins in the Ascomycota. While this class is not present outside the Ascomycota, VIVID homologs do have a large impact on mediating light responses so they are not excessive and wasteful. While the effects of ZtVVD on general growth and

development of *Z. tritici* are fairly minimal, this gene has a large effect on its pathogenicity. Further analyses are required to completely elucidate the reasons for this lack of pathogenicity, but it is still a curious response worth further investigation.

CHAPTER 4. ROLE OF A PHOTOLYASE IN GROWTH AND STRESS TOLERANCE OF ZYMOSEPTORIA TRITICI

4.1 Introduction

The Ascomycete fungus *Zymoseptoria tritici* (syn. *Mycosphaerella graminicola*, *Septoria tritici*) is a major pathogen of wheat (*Triticum* spp.), causing the disease Septoria tritici blotch (STB). This disease can cause up to 50% yield loss in epidemic years, and a majority of fungicides used on wheat in the EU are used in an attempt to control *Z. tritici* (Fones and Gurr 2015; Torriani et al. 2015). There are two primary methods used to control this pathogen: chemical fungicides and resistant cultivars (Ponomarenko et al. 2011). Currently, two classes of fungicides are effective against *Z. tritici*, the DMI (demethylation inhibitor) and the SDHI (succinate dehydrogenate inhibitor) fungicides. Resistance to many of these compounds is common in *Z. tritici*, as a rapid sexual reproduction cycle leads to a single resistant allele quickly spreading in the population, especially under selective pressure (Fraaije et al. 2005; McDonald and Mundt 2016; Neddaf et al. 2017; Kema et al. 2018). Resistance to the DMI fungicides is slowly spreading in *Z. tritici* populations; pockets of resistance to the SDHI fungicides have been found, but are not widespread (Fraaije et al. 2007, 2012; Stammler et al. 2008; Estep et al. 2015; Wieczorek et al. 2015; Dooley et al. 2016; Rehfus et al. 2018; Yamashita and Fraaije 2018). Resistant cultivars typically do not offer complete control, as known resistance genes, both qualitative and quantitative, do not protect against all strains of *Z. tritici* (Brown et al. 2015). As with chemical control, the frequent sexual reproduction of the fungus facilitates *Z. tritici* overcoming the host resistance, often very quickly (Ponomarenko et al. 2011; McDonald and Mundt 2016; Borg et al. 2018; Kristoffersen et al. 2020). Control of this disease is not currently stable.

Light is a very important environmental cue and stressor for fungi. UV light in particular can damage fungal cells in two ways: by forming reactive oxygen species (ROS); and by causing lesions in DNA that give rise to mutations, and possibly lead to death of the cells. There are three major lesion types caused by UV radiation to fungal DNA, two of which are common. The most common is a cyclopyrimidine dimer (CPD) lesion, where two adjacent pyrimidines bond together into a four-member ring (Figure 1) (Cadet et al. 2005). The next-most-common type is a 6-4 pyrimidine pyrimidone (6-4PP) lesion, where the 6-carbon in one pyrimidine and the 4-carbon in the next bond together (Figure 1) (Cadet et al. 2005). The least-common type is a Dewer dimer

lesion, which is formed when a 6-4PP lesion is irradiated with more UV energy, forming additional bonds (Figure 4.1) (Douki and Sage 2016).

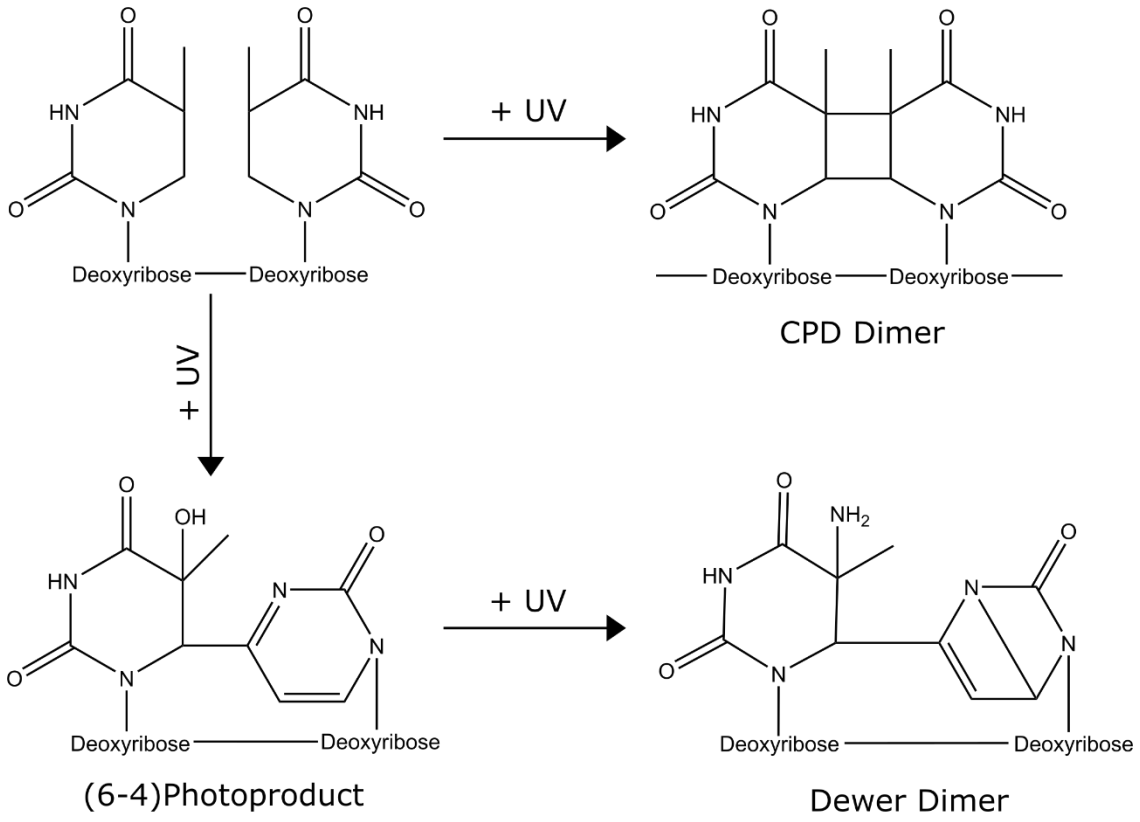


Figure 4.1 Diagram of DNA lesions caused by UV radiation, using thymine bases.

While the lesions initiated by UV light in the DNA of an organism are deleterious and cause mutations, most organisms possess repair mechaisms. One major method used by fungi to repair UV-damaged DNA employs the photolyase proteins. These proteins absorb blue light using a flavin-based chromophore and utilize the captured energy to repair the DNA lesion (Zhang et al. 2017a). Photolyases belong to the cryptochrome/photolyase class of proteins, which are ancient and found throughout the entire tree of life (Goosen and Moolenaar 2008; Sancar 2008; Mei and Dvornyk 2015). The cryptochrome proteins do not play any role in DNA repair but use a similar structure to regulate cellular functions. Cryptochromes have been shown to regulate reproduction

and secondary metabolism in response to light (Veluchamy and Rollins 2008; Froehlich et al. 2010; Castrillo et al. 2013; Cohrs and Schumacher 2017; Wang et al. 2017a)

Two types of photolyases are found in fungi. One repairs CPD lesions while the other repairs the (6-4)PP lesions. The other method of repairing DNA lesions is known as nucleotide excision repair (NER). In NER, the damaged bases are detected and replaced with undamaged bases by a series of twenty-five proteins (Prakash and Prakash 2000; Boiteux and Jinks-Robertson 2013; Petruseva et al. 2014). The first set of these proteins detects the lesion in the genome. The next set splits the DNA into single strands, and holds it open, so the third set can excise the damaged DNA. In the last step, a DNA polymerase complex that replaces the damaged nucleotides and closes the gap (Boiteux and Jinks-Robertson 2013). The general NER pathway repairs more than just UV damage, such as damage caused by chemical mutagens, but some species of fungi have an additional protein that specifically detects defects caused by UV (Fabre 1971; Yajima et al. 1995; Ishii et al. 1998; Yasui 2013; Verma and Idnurm 2013). The best known of these are the *Neurospora crassa* rad-18 and the *Schizosaccharomyces pombe* UVDE (Yajima et al. 1995; Yonemasu et al. 1997; Ishii et al. 1998; Inoue 2011; Yasui 2013).

These repair mechanisms are often activated by light in a process known as photoreactivation. When an organism is exposed to UV, it requires light to induce production of the genes required for repairing DNA lesions and coping with the associated damage. When a culture is exposed to UV light and then immediately transferred to darkness, it struggles to survive because of the UV damage to the DNA. This phenomenon has been well known for many years (Kelner 1949; Townsend 1961; Singh 1975; Yasui and Chevallier 1983; Schild et al. 1984; Chelico et al. 2006).

The genome of *Z. tritici* codes for one Cry-DASH cryptochrome, one photolyase, and two putative photolyases. This paper considers whether these four proteins have been identified correctly, and whether they are functional. The hypothesis that the known photolyase protein is required for photoreactivation, but not for regular growth, will also be tested using a deletion mutant.

4.2 Materials & Methods

4.2.1 Strains and Growth Conditions

The wild type strain and the strain used for gene disruptions was IPO323, the sequenced strain (Goodwin et al., 2011). All of the initial cultures were grown on potato dextrose agar (PDA) (Difco) at 19ºC in a darkened incubator. All the phenotyping plates were in a base of PDA with the phenotyping compound added, with the exception of the YSA (10 g of yeast extract (brand), 10 g of sucrose, 15 g of Bacto Agar per liter) and water agar (15 g of Bacto Agar). The light conditions were 16:8 day:night cycles under (brand) fluorescent bulbs, and full dark was achieved by wrapping the plates in aluminum foil.

4.2.2 Phylogenetic Tree

Sequences of known photolyase and cryptochrome genes were downloaded from the GenBank database at NCBI, and others were found via BLAST-P search using *N. crassa* phr (XP_964834) and cry (XP_965722), *M. oryzae* phr (XP_003708836), and *U. maydis* phr1 (XP_011392439), phr2 (XP_011388500), cry1 (XP_011387082), and cry2 (XP_011392255) gene sequencies as queries against Dikarya (Table 4.1) (Galagan et al. 2003; Kämper et al. 2006; Froehlich et al. 2010; Inoue 2011; Brych et al. 2016; Wang et al. 2017a). MEGA-X (version 10.1.7) was used to align the sequences via the integrated MUSCLE aligner, then a phylogenetic tree was generated from the aligned sequences using the maximum likelihood method, and 500 bootstrap replicates (Jones et al. 1992; Edgar 2004; Hurley et al. 2018). All default settings were used.

Table 4.1 List of all proteins used in the phylogenetic tree of cryptochromes and photolyases from fungi and *Arabidopsis thaliana*.

Species	Protein Name	Accession Number[a]
Alternaria alternata	Cry	XP_018380931.1
Alternaria alternata	Phr	XP_018383275.1
Arabidopsis thaliana	Phr	NP_566520.1
Arabidopsis thaliana	Cry3	NP_568461.3
Arabidopsis thaliana	Cry2	NP_171935.1
Aspergillus fumigatus	Phr1	XP_749910.1
Baudoinia panamericana	Cry/Phr_62992	XP 007673080.1
Baudoinia panamericana	Cry/Phr_67237	XP 007674658.1
Baudoinia panamericana	Cry/Phr_175264	XP 007671770.1
Baudoinia panamericana	Cry/Phr_53277 (partial)	XP 007678138.1
Bipolaris sorokiniana	Class I Phr	XP_007699765
Bipolaris sorokiniana	(6-4PP)Phr	XP_007703890
Bipolaris sorokiniana	Cry-DASH	XP_007694178
Cercospora zeae-maydis	Put. CPD Phr	KAF2216737.1
Cercospora zeae-maydis	Put. (6-4PP)Phr	KAF2212232.1
Cercospora zeae-maydis	Put. Cry	KAF2212150.1
Coniosporium apollinis	Cry/Phr_06495	XP_007782559.1
Coniosporium apollinis	Cry	XP_007778746.1
Coniosporium apollinis	Cry/Phr_05482	XP_007781702.1
Cordyceps militaris	Phr	XP_006665374
Cordyceps militaris	(6-4PP)Phr	XP_006667607
Cordyceps militaris	Cry-DASH	XP_006665996
Daldinia childiae	Phr	XP_033441191.1
Daldinia childiae	Cry	XP_033435737.1
Exophiala aquamarina	Phr	XP_013259262.1

[a] Accession numbers are from NCBI.

Table 4.1 continued

Exophiala aquamarina	Phr_04664	XP_013262406.1
Exophiala aquamarina	Cry	XP_013260571.1
Exophiala aquamarina	Phr_03481	XP_013264499.1
Fusarium graminearum	(6-4PP)Phr	XP_386941
Fusarium graminearum	CPD-Phr	XP_380973
Fusarium graminearum	Cry-DASH	XP_389028
Magnaporthe oryzae	(6-4PP)Phr	XP_003708836
Magnaporthe oryzae	Class I Phr	XP_003709552
Magnaporthe oryzae	Cry-DASH	XP_003720690
Neurospora crassa	Class I Phr	XP_964834
Neurospora crassa	Cry-DASH	XP_965722
Parastagonospora nodorum	(6-4PP)Phr	XP_001801925
Parastagonospora nodorum	Cry-DASH	XP_001798044
Penicillium digitatum	Phr	XP_014532134.1
Penicillium digitatum	Phr_62750	XP_014533501.1
Phialocephala scopiformis	Cry/Phr_679568	XP_018078773.1
Phialocephala scopiformis	Cry/Phr	XP_018075340.1
Phialocephala scopiformis	Cry	XP_018077771.1
Protomyces lactucaedebilis	Phr	ORY87110.1
Protomyces lactucaedebilis	phr-FAD	ORY86104.1
Protomyces lactucaedebilis	Cry/Phr	ORY83309.1
Pseudocercospora fijiensis	Cry/Phr_47206	XP 007925838.1
Pseudocercospora fijiensis	Cry/Phr_39565	XP 007922401.1
Pseudocercospora fijiensis	Cry/Phr_201892	XP 007922012.1
Pseudocercospora fijiensis	Cry/Phr_202046	XP 007922254.1
Pseudogymnoascus verrucosus	Cry/Phr_09125	XP_018126484.1
Pseudogymnoascus verrucosus	Cry/Phr_04549	XP_018131350.1
Puccinia graminis f. sp. tritici	Cry1	XP_003326630.1

Table 4.1 continued

Puccinia graminis f.sp. tritici	Phr1	XP_003324101.2
Puccinia graminis f.sp. tritici	Phr2	XP_003336224.2
Saccharomyces cerevisiae	phr1	CAA99718.1
Sclerotinia sclerotium	Phr	XP_001587047.1
Sclerotinia sclerotium	Cry	XP_001593735.1
Sporothrix schenckii	Phr	XP_016591947.1
Trichoderma reesei	(6-4PP)Phr	XP_006964927
Trichoderma reesei	CPD-Phr	XP_006965773
Trichoderma reesei	Cry-DASH	XP_006964352
Trichophyton rubrum	Phr	XP_003237491.1
Ustilago maydis	Phr2	XP_011388500.1
Ustilago maydis	Cry1	XP_011387082.1
Ustilago maydis	Phr1	XP_011392439.1
Ustilago maydis	Cry2	XP_011392255.1
Zasmidium cellare	Cry/Phr_25906	XP 033664609.1
Zasmidium cellare	Cry/Phr_60175	XP 033661163.1
Zasmidium cellare	Cry/Phr_69523	XP 033662598.1
Zasmidium cellare	Cry/Phr_63051	XP 033673220.1
Zymoseptoria tritici	Phr3	XP_003857369.1
Zymoseptoria tritici	Phr1	XP_003857772.1
Zymoseptoria tritici	Phr2	XP_003847594.1
Zymoseptoria tritici	Cry	XP_003853581.1

4.2.3 Nucleic Acid Manipulation

IPO323 genomic DNA was extracted using a Qiagen Plant DNeasy kit (cat #: 69104). A double-jointed PCR method of generating the replacement construct was used (Yu et al. 2004). The primer combinations ZtPhr1-A/ZtPhr1-B-HPH and ZtPhr1-C-HPH/ZtPhr1-D were used to generate the flanking regions from IPO323 genomic DNA (Table 4.2). These primers were designed using the genome sequence (Goodwin et al. 2011). Primers HPH-F/HPH-R were used to generate the hygromycin phosphotransferase (hph) segment of DNA from the plasmid pBHT2 (Table 4.2) (Mullins et al. 2001). These DNA fragments were purified using gel electrophoresis and extracted using a Qiagen QIAquick Gel Extraction kit (cat #: 28115) then combined without primers in a 1:3:1 molar ratio (Flank AB:hph:Flank CD) and joined using the double-jointed PCR method (Yu et al. 2004). The reaction was used directly in a third PCR with primers ZtPhr1-Nest-F/ZtPhr1-Nest-R, which contain restriction enzyme recognition sites for XbaI and HindIII, respectively (Table 4.2). The product was purified using an isopropanol-sodium acetate method and cloned into pBHT2 using the aforementioned restriction enzymes (XbaI: New England Biolabs cat #: R0145, HindIII: New England Biolabs cat #: R3104) and T4 DNA ligase (New England Biolabs cat #: M0202). This plasmid, designated pZPhr1, was cloned into competent TOP10 *E. coli* cells (Thermo Fisher cat #: C404010), then grown and extracted at higher concentration using a Qiagen QIAprep Spin Miniprep kit (cat #: 27104). Extracted pZPHR1 was transformed into competent *Agrobacterium tumefaciens* strain Agl1 using standard methods (Weigel and Glazebrook 2006).

Table 4.2 Primers used in transformation and confirmation of ZtPhr1 mutants in *Zymoseptoria tritici*. Underlined bases are the overlapping hygromycin phosphotransferase sequence, and lowercase letters are restriction enzyme recognition sites.

Primer Name	Primer Sequence (5' → 3') [ab]
HPH-F	GTCCAGGCGGTGAGCACAAAA
HPH-R	TTCCCGGTCGGCATCTACTCTATTC
ZtPhr1-A	CCTTGATGGGAGGGAGAGGA
ZtPhr1-B-HPH	TTTTGTGCTCACCGCCTGGAC TGCAAGAGGGATAGCTGCAT
ZtPhr1-C-HPH	GAATAGAGTAGATGCCGACCGGGAA GTCAGCGGCCTCGTACTATC
ZtPhr1-D	GATTGGTCGGAGGGCTGAAA
ZtPhr1-Nest-F	TCGTTACATCATCCCTtctagaGACTTGAAA
ZtPhr1-Nest-R	CTGCTGAGTGGTAGATaagcttATACTGTCG
ZtPhr1-Ver-F	CCTCTCCCTCCCATCAAGGA
ZtPhr1-Ver-R	TGCGCATAGGTGCATACAGA
ZtPhr1-Neg-F	GTTCCATCGCACGGGTATCA
ZtPhr1-Neg-R	ATACTGGTAGGTGTCCGCAT

[a] Underlined bases are the overlapping hygromycin phosphotransferase sequence.

[b] Lowercase letters are restriction enzyme recognition sites

4.2.4 Agrobacterium-Mediated Transformation and Verification

The *Agrobacterium tumefasciens* deletion mutations were generated as in Choi and Goodwin (2011) with the following modifications. The initial cultures were grown on YSA from glycerol stocks (25% glycerol) for four days. No filter paper was used after the *Agrobacterium* and *Z. tritici* were combined, and the cultures were transferred via pressing the face of one agar plate to the new one.

The hygromycin-resistant colonies were transferred to PDA containing 100 U/L of hygromycin and 200 μM cefotaxime, and subcultured to PDA containing only 100 U/L of hygromycin. The DNA was extracted as described above and used for verification PCR using the primers in Table 1. The ZtPhr1-Neg pair amplifies the ZtPhr1 gene, while ZtPhr1-Ver amplifies over the insert (Figure 4.2).

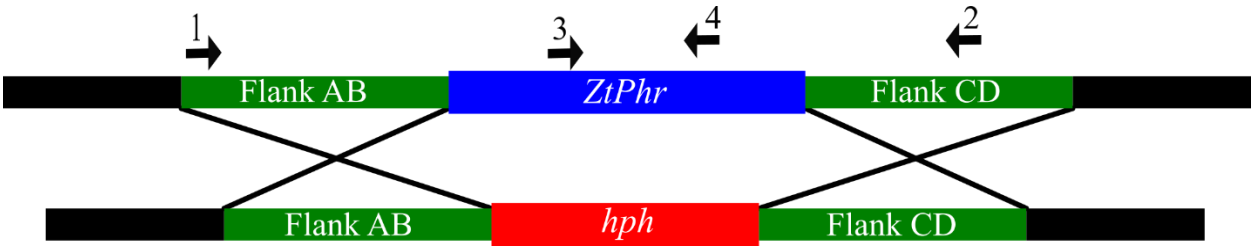


Figure 4.2 A schematic of the replacement construct and primer locations for the ZtPhr gene in the wheat pathogen *Zymoseptoria tritici*. Not to scale. 1 and 2 indicate the ZtPhr1-Ver primers, and 3 and 4 indicate the ZtPhr1-Neg primers.

4.2.5 Plate Phenotyping

Cells from a 4-day old plate of each strain were quantified and diluted to 10^7, 10^5, and 10^3 cells/mL. Two uL of each strain and concentration were inoculated on to a phenotyping plate and placed into the specified conditions. Plates placed in full dark were wrapped in aluminum foil. The plates were left to grow for 10 days before being scanned using an Epson photo 6800 flatbed scanner at 1200 dots per inch.

For photoreactivation tests, the plates were inoculated as before, and placed in a Stratagene UV Stratalinker 2400, face up with the plate covers removed. The plates were irradiated with 400, 800, or 1200 J/m^2 of 254 nm UV light. The covers were replaced, and the plates were sealed with Parafilm (Bemis). Half of the plates were wrapped in aluminum foil for the full dark condition. The plates were left for 10 days and then were scanned as described above.

4.2.6 Virulence Tests

Inoculum was made by scraping cells from a 4-day-old plate into water, adjusting to 10^7 cells/mL and adding Tween 20 to 0.1% immediately before inoculation. Fourteen-day-old wheat seedlings of the Taichung 29 cultivar were inoculated using a cotton swab and placed into a bag with water for 3 days to form a dew chamber, then were removed from the bag. At 21 and 28 days post inoculation (dpi), the first two leaves were scanned using an Epson Photo 6800 flatbed scanner at 1200 dots per inch.

4.3 Results

4.3.1 Phylogenetic Analysis

A total of 75 protein sequences from 24 species were downloaded from proteins studied and characterized as photolyase and cryptochrome proteins and based on the BLAST-P results. Most sequences were from Ascomycete species, with two Basidiomycete species represented, and the *Arabadopsis thaliana* cryptochrome and photolyase proteins were used as outgroups. Many of the fungal species were plant pathogens, but saprobes and animal pathogens were also represented (Figure 4.3 (Galagan et al. 2003; Dean et al. 2005; Nierman et al. 2005; Kämper et al. 2006; Cuomo et al. 2007; Hane et al. 2007; Bluhm and Dunkle 2008; Martinez et al. 2008; Goodwin et al. 2011; Duplessis et al. 2011; Zheng et al. 2011; Ohm et al. 2012; Martinez et al. 2012; Marcet-Houben et al. 2012; Wibberg et al. 2013; Guzmán-Moreno et al. 2014; Walker et al. 2016; Zapata et al. 2016; Zeiner et al. 2016; Derbyshire et al. 2017; Teixeira et al. 2017; Mondo et al. 2017; Palmer et al. 2018; Kim et al. 2020; Haridas et al. 2020).

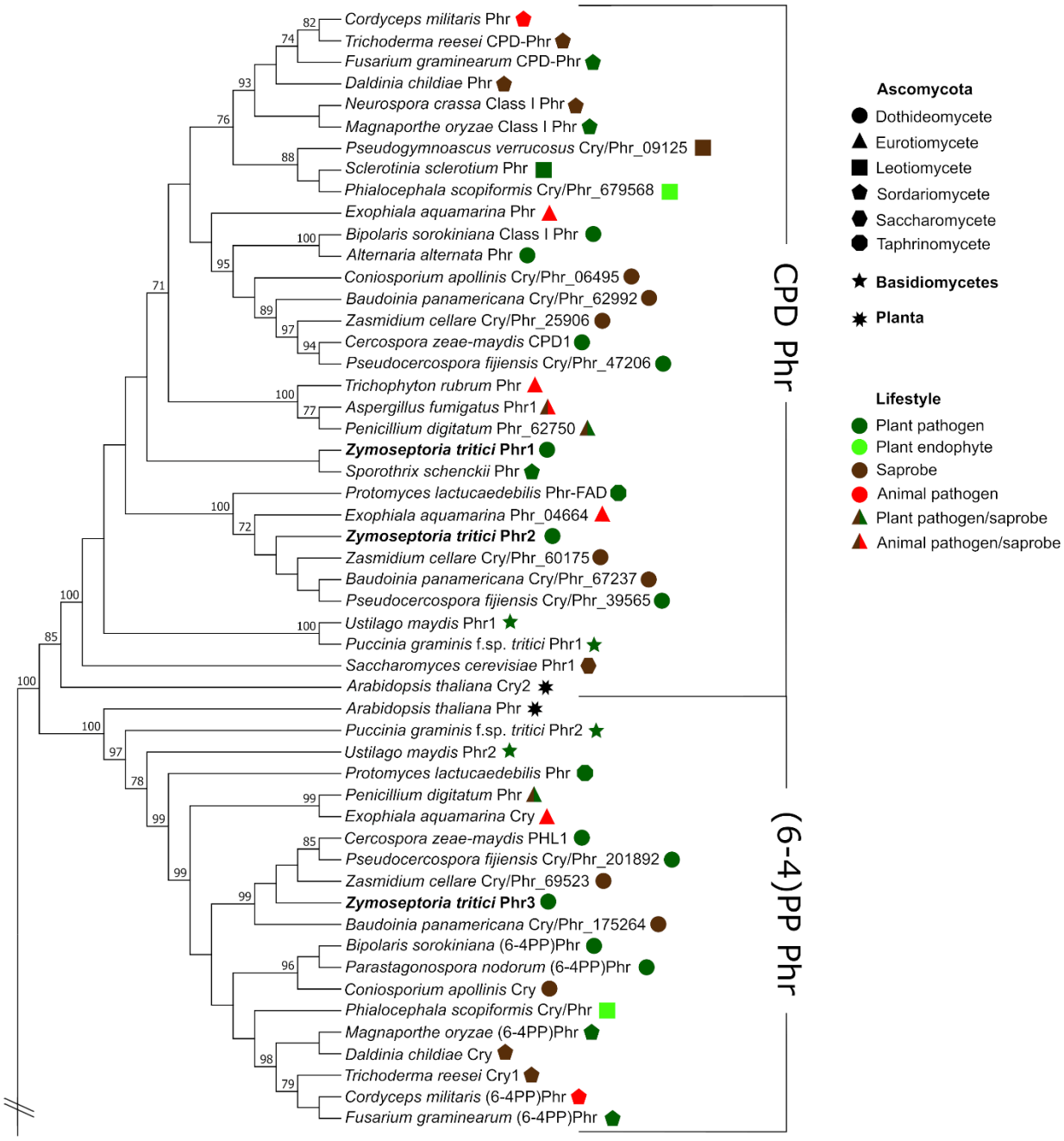
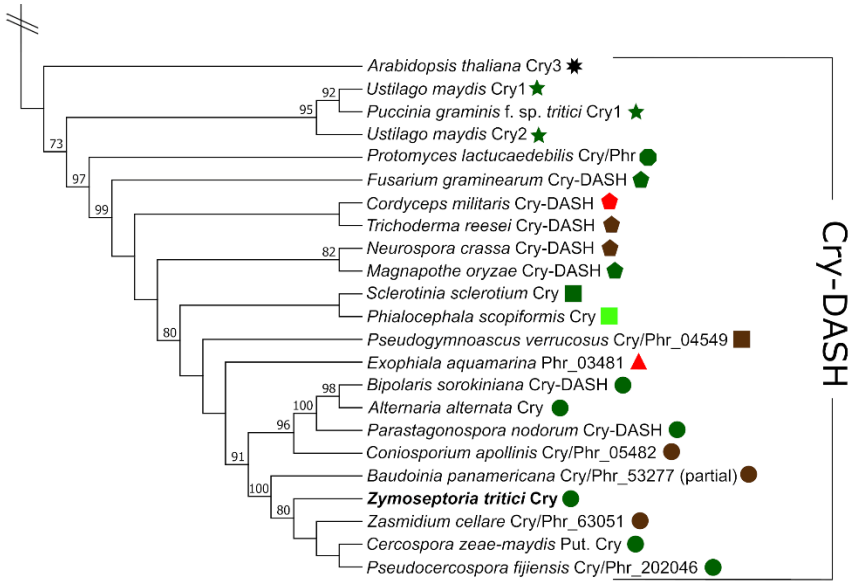


Figure 4.3 Phylogenetic tree of the cryptochrome/photolyase proteins from numerous fungal species across Dikarya with bootstrap values over 70% shown above the branches. The proteins are sorted into the three clades present in Dikarya.

Figure 4.3 continued



The various fungal photolyase and cryptochrome proteins were separated into three distinct clades with high bootstrap support, the CPD photolyases, the (6-4)PP photolyases, and the Cry-DASH cryptochromes. Of the three clades, the CPD photolyases are the most common. Thirty-one of the sequences were CPD photolyases, 19 were (6-4)PP photolyases, and 22 were Cry-DASH cryptochromes (Figure 4.3). All species but *Parastagonaspora nodorum* had at least one CPD photolyase present in their genome, and five species had two CPD photolyases. Four of the species with two CPD photolyases are Dothideomycetes, in the order Capnodiales, and the last is a black yeast from the Eurotiomycetes, *Exophiala aquamarina* (Figure 4.3). The Capnodiales is known to contain many sooty molds and other melanized fungi also known as black yeasts. The additional CPD photolyase and the high amounts of melanin may indicate that these fungi require additional defenses against UV. While NER can also repair UV damage done to DNA, the photolyase proteins work the fastest, and require the least amount of input. NER requires a network of proteins to function, while photolyases require only the one. If an organism experiences a large amount of UV radiation, and thus a large number of lesions, most of which will be CPD lesions, the duplication of a CPD photolyase would aid in survival.

The genome of *Z. tritici* contains genes coding for two CPD photolyases, one (6-4)PP photolyase, and 1 Cry-DASH cryptochrome (Table 4.3). The presence of an additional CPD photolyase is corroborated by the fact that *Z. tritici* is a member of the Capnodiales and is highly melanized under stress, both conditions that seem to correlate with an additional CPD photolyase found in the genome of a fungus. The amino acid residues related to methenyltetrahydrofolate (MTHF) and flavin binding are well conserved from the three model photolyase proteins (Figure 4.4). In many cases, where the amino acids are not identical, they are replaced with similar amino acids that have a high likelihood of functioning in the same fashion as the residues in the model species (Figure 4.4). *ZtPhr3* does not align as well as *ZtPhr1* and *ZtPhr2*, however that is because the three model photolyase proteins are CPD photolyases, and *ZtPhr3* is from the divergent lineage (6-4)PP photolyases (Park et al. 1995). These data indicate that the two CPD photolyases are likely to be functional photolyase proteins; however, it does not give us any definitive answers on the functionality of *ZtPhr3*.

Table 4.3 Summary information about the four cryptochrome/photolyase genes present in the genome of the wheat pathogen *Zymoseptoria tritici*.

Gene Name	Clade	Chromosome	Start	Stop
ZtCry	Cry-DASH	4	363577	365649
ZtPhr1	CPD Phr	1	379302	381080
ZtPhr2	CPD Phr	13	595339	597006
ZtPhr3	(6-4)PP Phr	1	2851232	2853406

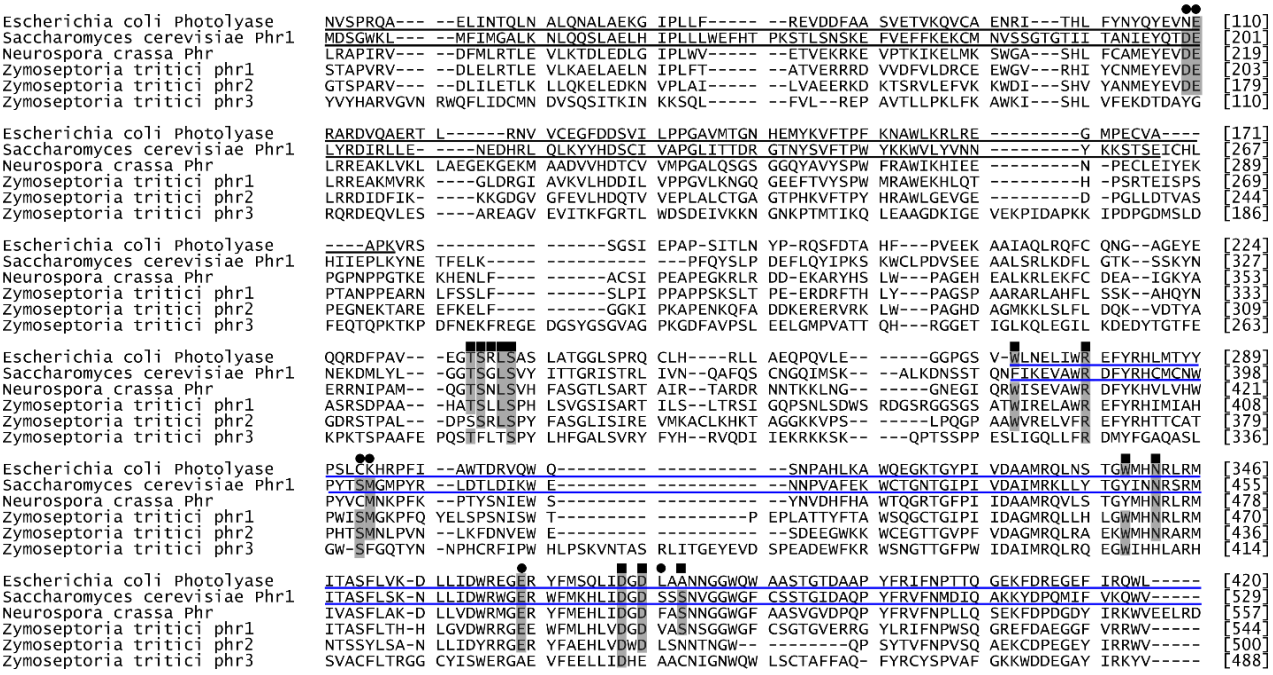


Figure 4.4 An alignment of the photolyase proteins from *Escheria coli*, *Saccharomyces cerevisiae* and *Neurospora crassa*, and the three photolyase proteins from *Zymoseptoria tritici*. Amino acids marked with a circle interact with the methenyltetrahydrofolate chromophore, and amino acids marked with a square interact with the flavin chromophore. Highlighted amino acids indicate interaction sites where they are conserved. Bases underlined in black indicate the DNA photolyase domain, and blue underlined bases show the FAD binding domain (El-Gebali et al. 2019).

Comparison of *ZtPhr3* to known functional (6-4)PP photolyases gives a better indication of the likelihood of functionality than comparing to CPD photolyases. When aligned with the (6-4)PP photolyase from *C. zeae-maydis*, *PHL1*, the two proteins are 70% identical (Bluhm and

Dunkle 2008). As *C. zeae-maydis* is closely related to *Z. tritici*, their lack of divergence is not surprising; however, the high similarity indicates a high level of conservation. This is also supported by a comparison to the *Trichoderma reesei* (6-4)PP photolyase, *Cry1* (Guzmán-Moreno et al. 2014). This fungus is much more distantly related, yet the proteins are still highly conserved, with 62.8% of amino acids identical. This indicates that there is a reasonable likelihood that *ZtPhr3* is a functional (6-4)PP photolyase.

4.3.2 Effect on Growth

The deletion of *ZtPhr1* does not lead to any differences in growth radius or general growth characteristics of *Z. tritici*. The production of melanin appears to be the same between the wild type IPO323 and the ΔZtPhr1 strain, as does the production of aerial hyphae and yeast-like versus hyphal growth. There are slight differences in growth characteristics between the light and the dark growth on PDA, in that the dark-grown *Z. tritici* has a greater number of aerial hyphae that form, giving it a fuzzier appearance than those grown in typical day:night cycles (Figure 4.5).

ΔZtPhr1 does not show any differences in single-cell germination. The branching pattern of the hyphal strains appeared similar regardless of the presence of ZtPhr1 (Figure 4.6).

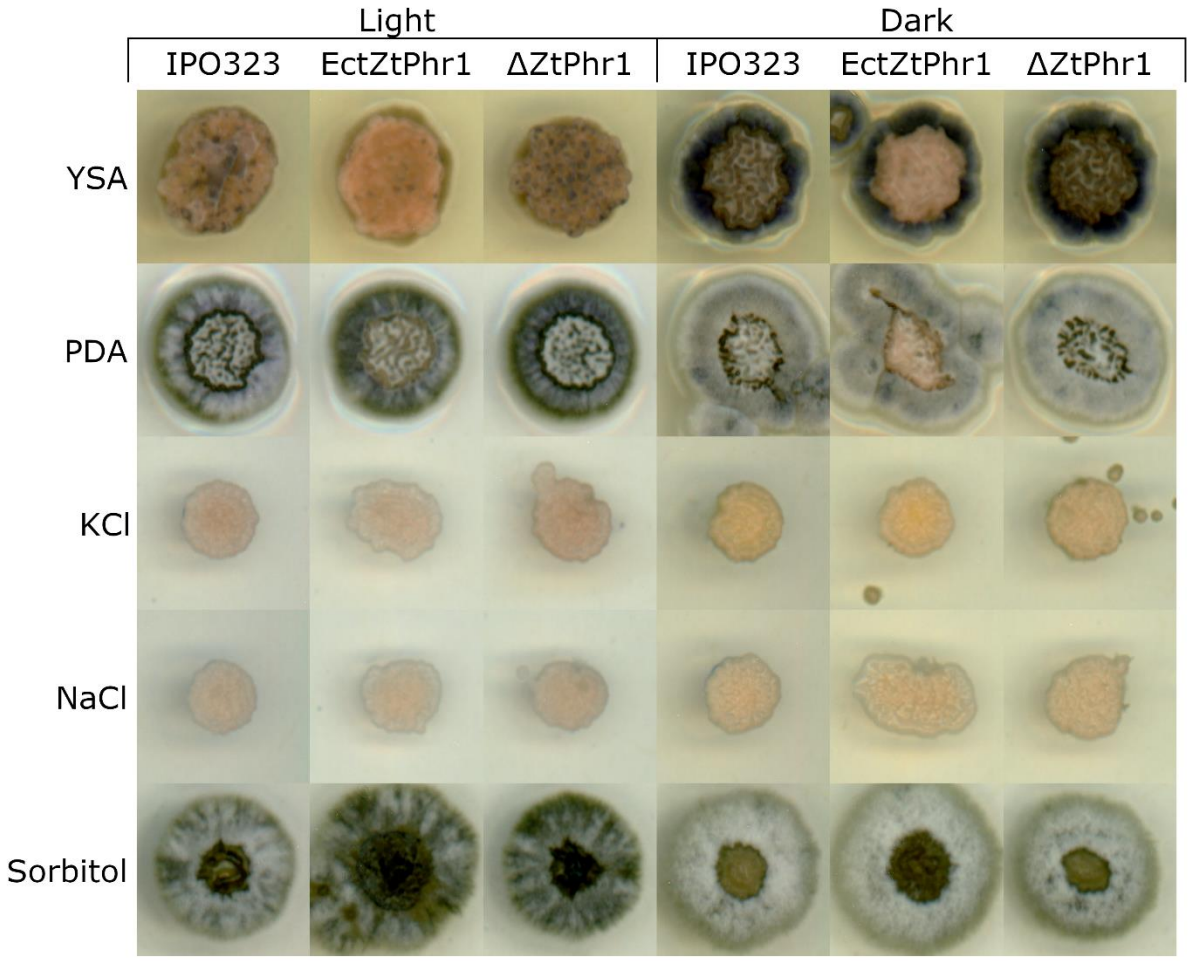


Figure 4.5 The wild type (IPO323), ectopic mutant (Ect) and deletion mutant (Δ) strains of the CPD photolyase gene *ZtPhr1* of the wheat pathogen *Zymoseptoria tritici* grown on media indicated on the left, under light conditions as shown at the top. The osmotic compounds were all included at a concentration of 1 M. Concentration of the suspension shown is 10^7 cells/mL; other concentrations gave similar results.

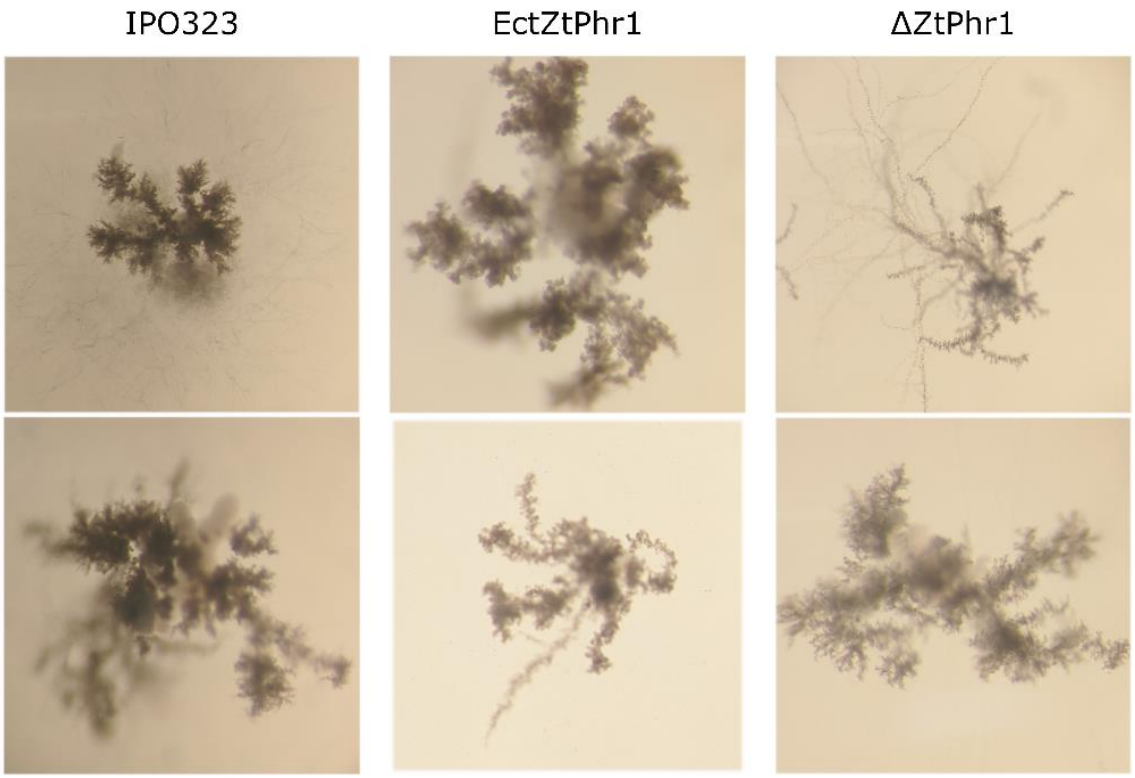


Figure 4.6 The colonies resulting from a single cell of the wild type (IPO323), ectopic mutant (Ect) and deletion mutant (Δ) strains of the CPD photolyase gene ZtPhr1 of *Zymoseptoria tritici* when grown on water agar for 30 days. All colonies were grown under identical conditions.

4.3.3 Stress Responses

ZtPhr1 deletion mutants show no difference in survivability under osmotic stresses. There also is no difference in growth characteristics when ΔZtPhr1 is grown under these osmostic stress conditions compared to IPO323 (Figure 4.5). However, the addition of 1M sorbitol does lead to additional melanization when exposed to day:night cycles, as opposed to those grown in full darkness in the wild type and in ΔZtPhr1 (Figure 4.5).

ΔZtPhr1 shows no change in survivability when under temperature stress, either high or low. There is also no sign of growth characteristics changing at different temperatures (Figure 4.7).

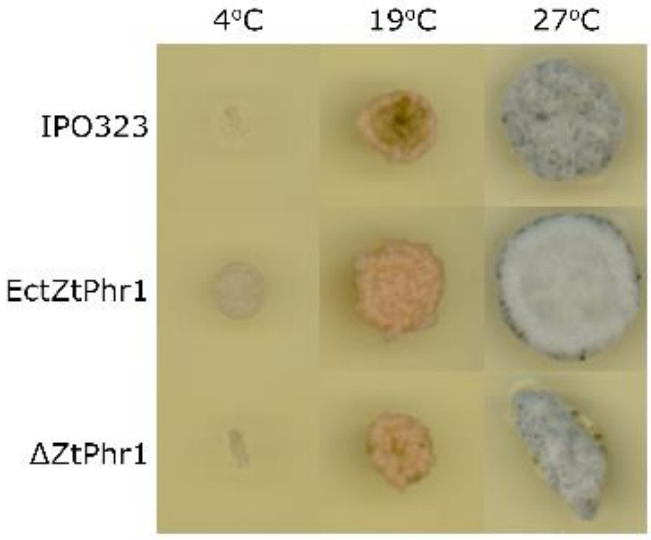


Figure 4.7 The wild type (IPO323), ectopic (Ect) and deletion (Δ) mutant strains of the *ZtPhr1* gene of *Zymoseptoria tritici* when grown at the temperatures shown at the top. Concentration shown is 10^7 cells/mL; other concentrations gave similar results.

Nutrient stress does not show any differences as well. When grown on water agar devoid of nutrients, the growth patterns were the same (Figure 4.8). There was also no change between the colonies grown in day:night cycles and those grown in the dark.

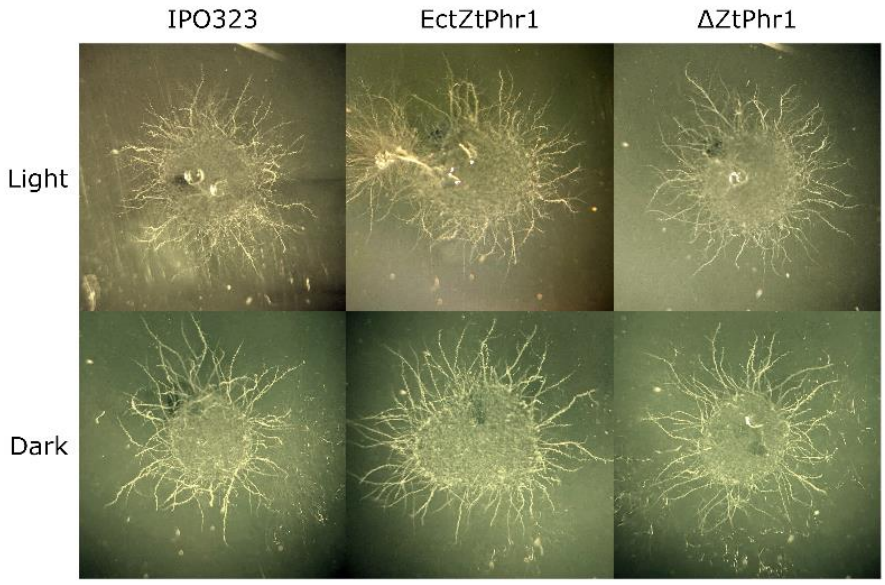


Figure 4.8 The wild type (IPO323), ectopic mutant (Ect) and deletion mutant (Δ) strains of the CPD photolyase gene *ZtPhr1* of the wheat pathogen *Zymoseptoria tritici* grown on water agar at a concentration of 10^7 cells/mL.

4.3.4 Affect on Photoreactivation

The deletion of *ZtPhr1* does not affect the survival of *Z. tritici* when irradiated by UV energy (Figure 4.9). There appears to be no phenotypic difference between the wild type IPO323 and the ΔZtPhr1 mutant. As *Z. tritici* has an additional CPD photolyase, it is possible that *ZtPhr2* is acting in a redundant fashion, making up for the lack of *ZtPhr1*.

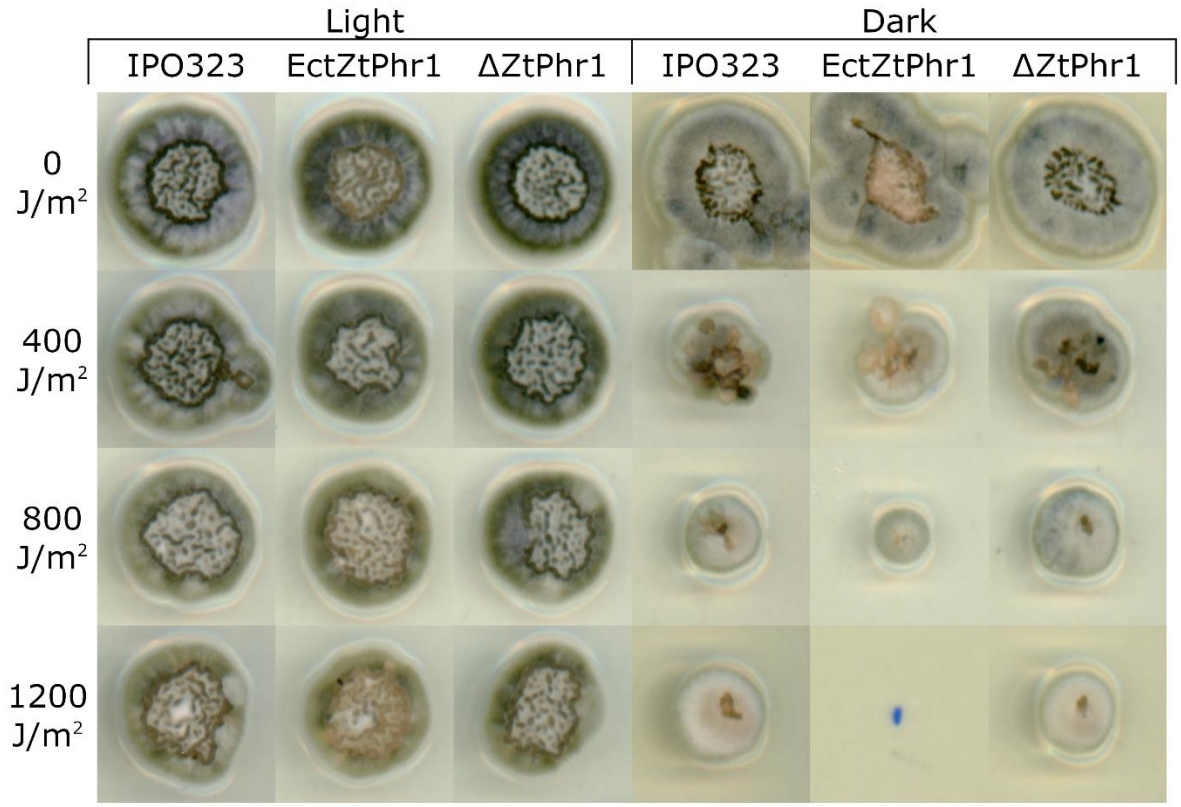


Figure 4.9 The three strains of *Zymoseptoria tritici* after irradiation with the amount of UV energy to the left and placed in the light conditions indicated above immediately after. Concentration shown is 10^7 cells/mL.

4.3.5 Oxidative Stress

The deletion mutant of *ZtPhr1* does not cause any change in how well *Z. tritici* survives oxidative stress. The ΔZtPhr1 strain and the IPO323 had similar growth radii and survival. The deletion of *ZtPhr1* causes additional melanization in the mutant, especially when the cultures were incubated in full dark (Figure 4.10). Melanin can act as an antioxidant to detoxify ROS, including H_2O_2, so this is an understandable reaction.

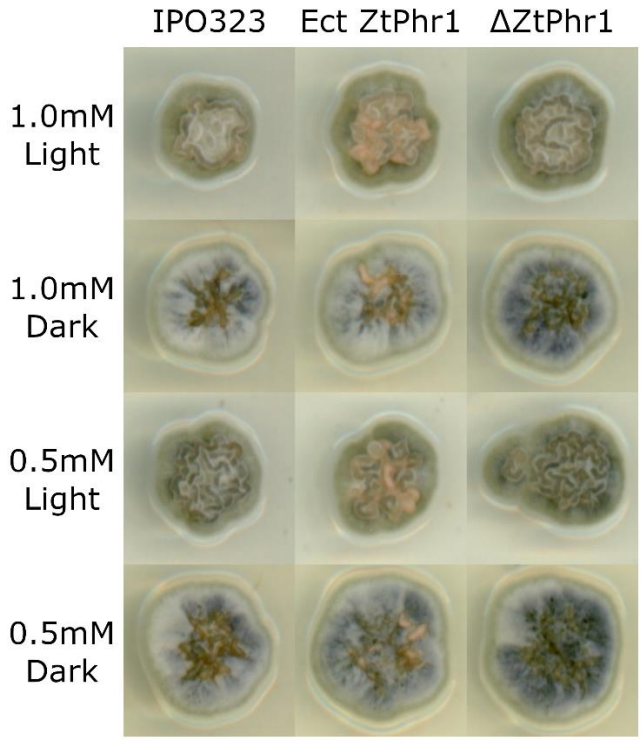


Figure 4.10 The wild type (IPO323), ectopic mutant (Ect) and deletion mutant (Δ) strains of the CPD photolyase gene *ZtPhr1* of *Zymoseptoria tritici* grown on the concentrations of H_2O_2 and light conditions indicated on the left. Concentration shown is 10^5 cells/mL.

4.3.6 Virulence

When each strain of *Z. tritici* were inoculated onto highly susceptible wheat plants, the infection pattern and intensity of the deletion mutant was the same as the wild type IPO323 and the ectopic mutant. All three strains caused necrosis of the leaf tissue and showed pycnidia formation on the inoculated leaves (Figure 4.11).

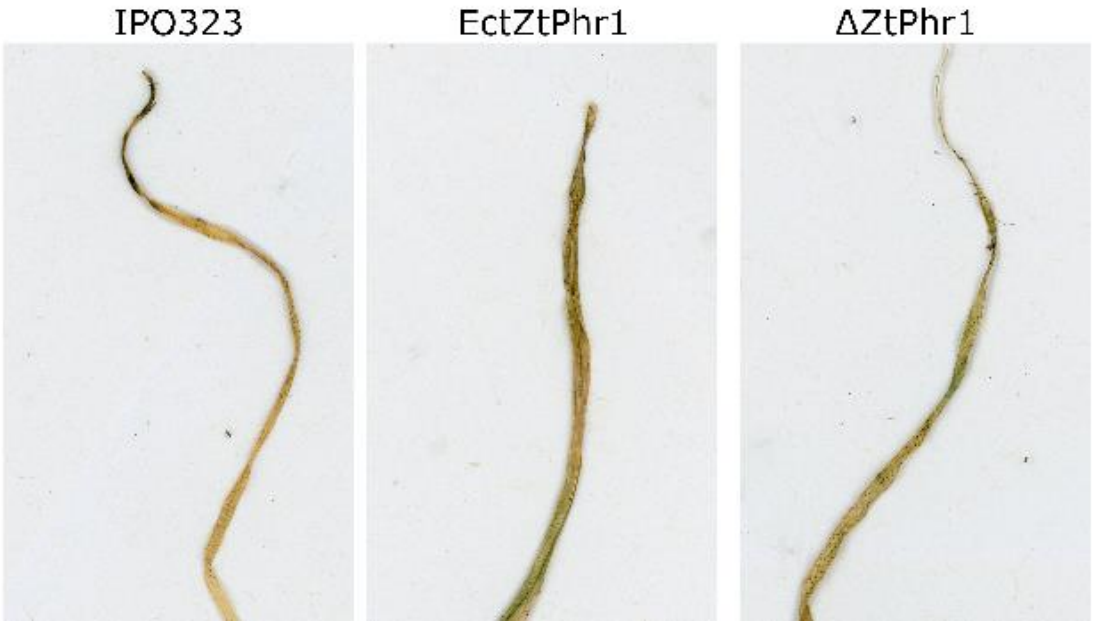


Figure 4.11 Wheat leaves of the highly susceptible cultivar Taichung 29 inoculated with the indicated strain of *Zymoseptoria tritici* at 28 days post inoculation. IPO323: wild type (untransformed) strain; Ect: ectopic mutant; and Δ: deletion mutant of the CPD photolyase gene *ZtPhr1*.

4.4 Discussion

Cryptochromes and photolyases are part of an ancient and well-conserved class of proteins. They are found throughout life, from bacteria and archaea to fungi and animals. The three primary clades known in the Dikarya subkingdom of fungi correspond to those found here: the class I CPD photolyases, the (6-4)PP photolyases, and the Cry-DASH cryptochromes (Bayram et al. 2008a; Bluhm and Dunkle 2008; Chaves et al. 2011; Mei and Dvornyk 2015; Cohrs and Schumacher 2017; Wang et al. 2017a). Additional clades exist in other realms of life; however, these are the only three clades found in Dikarya (Chaves et al. 2011; Mei and Dvornyk 2015).

The lack of notable phenotypes in the ΔZtPhr1 strain of *Z. tritici* is reasonably expected. Photolyase proteins have one primary function: repairing DNA lesions in an organism's genome. The lack of change in photoreactivation and UV sensitivity is not unexpected either. *Z. tritici* contains numerous redundant pathways and proteins to repair DNA lesions caused by UV light. As found in the phylogenetic analysis, *Z. tritici* not only contains a (6-4)PP photolyase, but an additional CPD photolyase, and an investigation into the genome indicates that *Z. tritici* also has

a homolog of the *N. crassa mus-18* and *S. pombe UVDE* genes (Yajima et al. 1995; Yonemasu et al. 1997; Ishii et al. 1998; Inoue 2011; Yasui 2013). These genes code for proteins that detect UV damage to DNA specifically and guide the proteins in the NER pathway to effect its repair (Fabre 1971; Yajima et al. 1995; Yonemasu et al. 1997; Ishii et al. 1998). With the other methods *Z. tritici* possesses to repair UV-damaged DNA, it is not surprising that there was no change in UV sensitivity when only a single gene was knocked out. However, it also does not show that ZtPhr1 is a functional photolyase.

While the primary function of photolyases is to repair UV-damaged DNA, some photolyases have additional photoreceptor-like regulatory roles in cells. One photolyase with regulatory function is the photolyase cryA from *Aspergillus nidulans*. A functional protein in the CPD photolyase clade, it also can repress sexual development (Bayram et al. 2008a). Another photolyase that displays regulatory function is *PHL1* from *Cercospora zeae-maydis*. This functional (6-4)PP photolyase represses conidiation and slightly induces biosynthesis of the photoactivated toxin cercosporin (Bluhm and Dunkle 2008). Thus, while *ZtPhr1* does not show any major regulatory function, only showing minor changes in melanin production, it is possible that the other photolyase genes, especially *ZtPhr3*, do have this role.

This study, while not yielding many obvious results on its own, does open questions regarding *Z. tritici* and DNA repair. The immediate question is about the ectopic mutant used in this study. While the ΔZtPhr1 strain did not show any differences in DNA repair after UV irradiation or in general growth, the EctZtPhr1 strain did, showing a sensitivity to UV radiation when incubated in the dark and changed levels of melanization under various conditions. This strain has the *hph* gene replacement inserted in a random place in the genome instead of replacing a known gene, and the question is: where is it inserted to cause this phenotype? The phenotypes of the three strains indicate that it is the insertion location and not the *hph* gene causing these phenotypes. If it were the *hph* gene, the deletion and ectopic mutants would have similar phenotypes, and not the wild type and deletion mutant.

Another question arises from a previous study which found that *ZtPhr2* and *ZtPhr3* were differentially expressed under different light conditions, while *ZtPhr1* was not (McCorison and Goodwin 2020). In the future, it would be worth examining whether *ZtPhr2* and *ZtPhr3* have regulatory functions similar to *A. nidulans cryA* and *C. zeae-maydis PHL1* as well as assist in photorepair. This could be easily done using a deletion mutant of each protein and examining if

there are differences in development and metabolism. It also would be worth investigating whether *ZtPhr1* and *ZtPhr2* are both required for full repair functionality using a deletion mutant of *ZtPhr2*, and a double deletion mutant of both CPD photolyases. As indicated by the phylogenetic analysis of the four photolyase/cryptochrome genes present in *Z. tritici*, the functions of these proteins likely overlap. Another future direction for research would be examining the UV-specific NER pathway, using a deletion mutant to examine if the *UVDE* homolog is functional. A double deletion mutant of the two CPD photolyases would show how these two proteins compensate for each other. There are many future pathways available in this field of study, and this only illuminates them.

4.5 Conclusions

The *Z. tritici* genome contains two CPD photolyases (*ZtPhr1* and *ZtPhr2*), one (6-4)PP photolyase (*ZtPhr3*), and one Cry-DASH cryptochrome (*ZtCry*). The photolyase proteins appear to be functional from analyses done here; however, the disruption of *ZtPhr1* showed no major differences from wild type. This indicates that the redundant CPD photolyase and the additional pathways *Z. tritici* has available to cope with UV damage can make up for the lack of one CPD photolyase. Further studies are required to fully shed light on the methods *Z. tritici* uses to cope with UV damage to DNA, and the precise functions of the other cryptochrome/photolyase proteins.

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VITA

Education

June 2013 - Present Ph.D. in Plant Pathology, Purdue University

Expected Graduation: December 2020

Current GPA: 3.65

Advisor: Dr. Stephen Goodwin

May 2013 - B.S. in Biochemistry and Biotechnology, Minnesota State University Moorhead

Minors: Biology and Chemistry

GPA: 3.66

Research Experience

Research Assistant, Department of Botany and Plant Pathology, Purdue University

June 2013 - Present: Investigating the photobiology of *Zymoseptoria tritici* (syn: *Mycosphaerella graminicola*, *Septoria tritici*) and how the photobiology affects the pathogenicity and virulence of the pathogen

Research and Development Intern, Dow AgroSciences, Indianapolis, Indiana

May 2015 - August 2015: Developing a high throughput method to transform *Zymoseptoria tritici*

Undergraduate Research, Department of Biology, Minnesota State University Moorhead

August 2012 - May 2013: Helped to develop a qPCR method to determine the genotype of transgenic maize, specifically the gene copy number, using a southern blot method to validate the qPCR results.

August 2011 - April 2012: Studied the levels of polyphenol oxidase present in *Cucumis sativus* cells at various time points after induction of systemic acquired resistance with salicylic acid using a spectrophotometric method to determine the rate at which catechol was being oxidized

Sept. 2009 - April 2010: Studied the levels of phenolic acids present in the *Cucumis sativus* cell walls at various time points after induction of systemic acquired resistance with salicylic acid using a spectrophotometric method to determine the concentrations of various acids in a cell wall extract.

Grants

Fall 2011 - College of Social and Natural Science Undergraduate Research Grant ($700.00) Minnesota State University Moorhead.

Presentations

Cassandra McCorison, Stephen B. Goodwin. Effect of the light sensing gene VIVID on growth and pathogenicity of *Zymoseptoria tritici.* Plant Health 2019.

Cassandra McCorison, Stephen B. Goodwin. The wheat pathogen Zymoseptoria tritici senses and responds to different wavelengths of light. International Conference of Plant Pathology 2018.

Cassandra McCorison, Javier Delgado, Cruz Avila-Adame, Gary Gustafson

Cassandra McCorison. Proposal to study the effects of varying light conditions on the pathogenicity of *Mycosphaerella graminicola.* Poster at Botany and Plant Pathology Poster Session 2013.

Cassandra McCorison, Christina Poudyal, Chris Chastain. Developing an Accurate qPCR Method for Determining the Genotype of Genetically Modified Maize. Poster at Minnesota State University Student Academic Conference 2013.

Cassandra Anderson, Andrew Marry. An investigation into potential alterations of polyphenol oxidase activity in *Cucumis sativus* seedlings following the abiotic induction of systemic acquired resistance. Poster at Minnesota State University Student Academic Conference 2012.

Omar Abdi, Sondra McGregor, Cassandra Anderson, Andrew Marry. Analysis of phenolic acids upon induction of systemic acquired resistance in *Cucumis sativus*. Poster at Minnesota State University Student Academic Conference 2010.

Teaching Experience

Fall 2017 - Teaching Assistant Department of Botany and Plant Pathology, Purdue University

BTNY 110 - Introduction to Plant Sciences: Taught and graded laboratory classes.

Spring 2013 - Teaching Assistant, Department of Chemistry, Minnesota State University Moorhead

CHEM 405 - Biochemistry 2: Assisted with preparation of reagents.

CHEM 105 - Crime Scene Science: Assisted with preparation and grading.

July 2012 - Summer Camp Assistant at Discovery Science Camp, Minnesota State University Moorhead: Assisted in teaching children the science of crime scene forensics.

Fall 2011 - Teaching Assistant, Department of Biology, Minnesota State University Moorhead BIOL 115 - Organismal Biology: Assisted with teaching the laboratory class.

Leadership

Sept. 2019 - present: Multimedia Manager of Botany and Plant Pathology Graduate Student Organization

Sept. 2015 - May 2016: Member of Purdue Graduate Student Government Committee Legislative and Strategic Planning.

Sept. 2012 - April 2013: Co-President of the Chemistry and Biochemistry Club, Minnesota State University Moorhead.

PUBLICATIONS

McCorison CB, Goodwin SB (2020) The wheat pathogen *Zymoseptoria tritici* senses and responds to different wavelengths of light. BMC Genomics 21. doi: 10.1186/s12864-020-06899-y

Goodwin, S.B., McCorison, C.B., Cavaletto, J.R., Culley, D.E., LaButti, K., Baker, S.E., Grigoriev, I. V., 2016. The mitochondrial genome of the ethanol-metabolizing, wine cellar mold *Zasmidium cellare* is the smallest for a filamentous ascomycete. Fungal Biol. 120, 961–974. doi:10.1016/j.funbio.2016.05.003