Identification of Aspergillus Fungal Resistance Factors in a Plant Model System

Teresa C. De Sitter
Western Washington University, desittt@wwu.edu

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IDENTIFICATION OF ASPERGILLUS FUNGAL RESISTANCE FACTORS IN A PLANT MODEL SYSTEM

By

Teresa C. De Sitter

Accepted in Partial Completion of the Requirements for the Degree Master of Science

Kathleen L. Kitto, Dean of the Graduate School

ADVISORY COMMITTEE

Co-chair, Dr. Jeff C. Young

Co-chair, Dr. Marion Brodhagen

Dr. Gregory O’Neil
MASTER’S THESIS

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Teresa C. De Sitter
December 20, 2016
IDENTIFICATION OF *ASPERGILLUS* FUNGAL RESISTANCE FACTORS IN A PLANT MODEL SYSTEM

A Thesis
Presented to
The Faculty of
Western Washington University

In Partial Fulfillment
Of the Requirements for the Degree
Master of Science

By

Teresa C. De Sitter
December 2016
ABSTRACT

Aspergillus flavus is a saprophytic, mycotoxigenic fungus that contaminates agriculturally important seeds with the potently toxic and carcinogenic secondary metabolite, aflatoxin. Seed infection by fungi is often prevented by intact seed coats. Although Arabidopsis thaliana is naturally resistant to Aspergillus infection, certain mutants in the flavonoid biosynthetic pathway are compromised in seed coat integrity. We hypothesized that these mutants might also permit Aspergillus infection. To that end, we systematically tested infectibility of mutants in the flavonoid biosynthetic pathway to identify those lacking resistance to Aspergillus fungal infection. Susceptible seeds included those mutated in the genes encoding for synthesis of the first flavonoid pathway precursor, chalcone, through leucocyanidin (CHS, F3’H, and DFR), indicating that the requisite compound is either leucocyanidin or a derivative of that compound. While preliminary observations suggested that older chs seeds might be more susceptible to A. nidulans than younger seeds, an experiment testing infectibility of seeds harvested at specific ages failed to reproduce the infection rates previously observed. Further investigation revealed that chs seed batches dominated by non-viable seeds are more infectible, as expected from a saprophytic fungus. A novel finding was that chs seeds formed during the final weeks of the parent plant’s development are more highly susceptible to A. nidulans. Our results suggest that wildtype Arabidopsis seeds have a barrier to infection, which may be either mechanical or chemical.
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# TABLE OF CONTENTS

ABSTRACT .................................................................................................................................................. iv

ACKNOWLEDGMENTS .......................................................................................................................... v

LIST OF FIGURES ..................................................................................................................................... vii

LIST OF TABLES ....................................................................................................................................... viii

INTRODUCTION ......................................................................................................................................... 1-13

METHODS .................................................................................................................................................... 14-19

RESULTS ...................................................................................................................................................... 20-23

DISCUSSION ............................................................................................................................................... 24-32

CONCLUSION ............................................................................................................................................. 33-34

REFERENCES ............................................................................................................................................. 35-43

FIGURES AND TABLES ............................................................................................................................... 44-65
LIST OF FIGURES

Figure 1. The five cell layers on an *Arabidopsis thaliana* seed coat ......................................... 44
Figure 2. The flavan backbone and classes of flavonoids .......................................................... 45
Figure 3. The flavonoid biosynthetic pathway in *Arabidopsis* ................................................. 47-48
Figure 4. Levels of expression of the seventeen laccase genes in *Arabidopsis* ......................... 49
Figure 5. Molecular changes made by flavonoid modifying enzymes ........................................ 50
Figure 6. Ages of siliques along the branches of an *Arabidopsis* plant ..................................... 51
Figure 7. A two-model pathosystem: *Arabidopsis thaliana* and *Aspergillus nidulans* ........... 52
Figure 8. Infection of *Arabidopsis* seeds mutant in flavonoid pathway genes ......................... 53
Figure 9. Rates of *A. nidulans* infection of laccase mutant seeds (*lac15* and *lac5*) ............. 54
Figure 10. Rates of *A. nidulans* infection of *chs* mutant seeds of different ages ................. 55-56
Figure 11. Timecourse of *A. nidulans* infection of *Arabidopsis* Wt Col and *chs* seeds ...... 57
Figure 12. Comparison of *chs* seeds with different plant-care methods ................................... 58-59
Figure 13. Comparison of *chs* seeds formed at different developmental stages ................. 60-61
Figure 14. Comparison of *chs* seeds with different genetic backgrounds ............................... 62
Figure 15. Inhibition of *A. nidulans* in the presence of Wt Col aqueous seed extract ........... 63
Figure 16. Variability of *chs* seed infection, germination, and chemical inhibition .............. 64-65
LIST OF TABLES

Table 1. *Arabidopsis* flavonoid biosynthetic pathway proteins, genes, and seed lines .......... 46
INTRODUCTION

Aspergillus flavus infects crops:

Aspergillus flavus is a ubiquitous, filamentous, saprophytic fungus that infects oil-containing seeds, including maize, peanut, and cottonseed (reviewed by Amaike and Keller, 2011). The fungus produces many secondary metabolites, including aflatoxins (reviewed by Klich, 2007), which are the most carcinogenic naturally occurring molecules known (reviewed by Amaike and Keller, 2011). Low-level, long-term ingestion of aflatoxins causes numerous chronic medical problems ranging from immunosuppression to cancer; ingestion of high levels of aflatoxins is lethal (reviewed by Yu et al., 2005; Shephard, 2008). A. flavus thrives in the tropical and subtropical regions of the world with temperatures between 25-42˚C, and it proliferates in more temperate regions during times of excess heat and drought (reviewed by Amaike and Keller, 2011; reviewed by Payne, 1998).

A. flavus colonization and aflatoxin contamination can be initiated in the field prior to harvest, mainly via cracked and damaged seeds, while subsequent fungal growth is often enhanced during post-harvest crop storage due to environmental storage conditions. Drought can cause cracking of seed coats, which in turn permits ingress of A. flavus germinating spores and hyphae into embryonic tissues (Keller et al., 1994; Cotty and Jaime-Garcia, 2007). Wounds caused by insect boring can also provide an entrance point for A. flavus hyphae (for example, see Sétamou et al., 1997). A. flavus can also colonize maize kernels via external growth of fungal hyphae down the length of the silk (Marsh and Payne, 1984; reviewed by Payne, 1998). Cottonseeds can be infected when the fungus gains ingress via the nectaries and grows into the developing boll (Klich et al., 1984; Klich and Chmielewski, 1985). In peanut, whose seeds grow below ground, floral invasion is not a significant source
of preharvest *A. flavus* contamination. Instead, infection occurs primarily through direct penetrance of the pod (Cole *et al*., 1986). Post-harvest *A. flavus* infection, which is the primary cause of aflatoxin contamination worldwide, often results from improper storage practices. If cracked and wounded seeds are infected by *A. flavus* prior to harvest, the fungus will spread rapidly in storage facilities with higher moisture levels and warm temperatures (Cotty and Jaime-Garcia, 2007; Magan *et al*., 2003; Giorni *et al*., 2008; reviewed by Klich, 2007).

**Current management methods are not sufficient for preventing *A. flavus* infection and aflatoxin contamination:**

Efforts to manage *A. flavus* invasion and aflatoxin contamination of crops can target any of the three components involved in fungal infection: pathogen, environment, or host.

*Pathogen:* The *A. flavus* life cycle is not conducive to elimination of the ubiquitous soil pathogen. *A. flavus* forms sclerotia, which can survive for several years buried in the soil without a host (Wicklow *et al*., 1993). Under favorable conditions the sclerotia germinate to form mycelia that then form conidiophores. The powdery conidia are either dispersed by wind or insects and infect aboveground seeds, or are dispersed via rain and soil movement and infect belowground seeds (Cole *et al*., 1986; reviewed by Amaike and Keller, 2011). Since *A. flavus* resists most registered fungicides, an emerging method for prevention of aflatoxin contamination is inoculating fields with non-aflatoxigenic strains of *Aspergillus*, thereby introducing a near-isogenic competitor into the niche that is usually filled by aflatoxin-producing *A. flavus* strains. This method must be implemented with caution since the non-aflatoxigenic strains could potentially mutate or sexually recombine to revert to toxin-forming strains. Additionally, this method is controversial because even strains that do
not produce aflatoxins can cause ill effects in humans, such as allergic reactions to spores, aspergillosis (recalcitrant fungal infections usually from inhalation or introduction via wounds) and toxicity from ingestion of other mycotoxins (reviewed by Klich, 2007).

Environment: Current disease management practices focus on creating pre- and post-harvest environmental conditions that are not favorable to A. flavus infection and aflatoxin contamination. Preventative methods begin with controlled cultural practices in the field, including sufficient irrigation to prevent seed cracking, control of insect pests to prevent seed wounding, and immediate harvesting after the crop reaches maturity to prevent fungal growth under uncontrolled environmental conditions of the field. In storage facilities, environmental conditions such as moisture levels, temperature, and atmospheric composition must be controlled to prevent post-harvest infection. A. flavus can grow and produce aflatoxin in moisture contents as low as 8-12% and 17-19%, respectively (reviewed by Sanchis and Magan, 2004). Most storage facilities can only dehumidify to 14% moisture content, which reduces aflatoxin production but does not fully eliminate fungal growth (reviewed by Payne, 1998). Though the optimal temperature for A. flavus growth is between 25-42˚C, some strains can grow in temperatures as extreme as 12-48˚C (reviewed by Payne, 1998), making it difficult to maintain storage facilities that are cool enough to prevent A. flavus infection. Artificially raising atmospheric CO₂ levels in storage facilities to 25% can reduce A. flavus growth, but at least 50% CO₂ is required to reduce aflatoxin production, though the exact mechanism of CO₂ inhibition is not yet understood (Giorni et al., 2008). CO₂ comprises only 0.04% of the gaseous compounds found in dry air (Dlugokencky and Tans, 2016), and maintaining high CO₂ levels in storage facilities is not practical for most farmers around the world.
**Host:** Since managing the pathogen and the environment is not sufficient for fungal elimination, resistant plant crops provide another method for prevention of *A. flavus* infection and aflatoxin contamination. Several cultivars of both maize and peanut are naturally resistant to *A. flavus* infection (Kelley et al., 2012; Liang et al., 2006; reviewed by Fountain et al., 2015; reviewed by Brown et al., 1999) and are used by farmers in regions with high frequencies of contamination. Upregulation of stress-related genes, such as those involved in the biosynthesis of phenylpropanoids (Wang et al., 2016a), can aid in resistance to fungal infection and can specifically reduce aflatoxin contamination (Dolezal et al., 2014; Chen et al., 2004; Chen et al., 2006; Chen et al., 2010; Guo et al., 2006; Guo et al., 2008).

Understanding the specific genetic differences between plants that are susceptible to *Aspergillus* fungal infection and those that are resistant is a crucial step in creating an effective method for preventing *A. flavus* invasion.

**A two-model pathosystem could help identify a mode of seed resistance to *A. flavus:***

*Aspergillus nidulans* is a model fungus. This fungus has been extensively studied and its entire genome was sequenced in 2001 (Galagan et al., 2001). Unlike most *Aspergillus* species, *A. nidulans* can undergo sexual recombination during formation of ascospores (Pontecorvo et al., 1953). While it contains most of the gene cluster responsible for aflatoxin production, *A. nidulans* only produces the penultimate precursor of the aflatoxin biochemical pathway, the mycotoxin sterigmatocystin (Keller and Adams 1995).

*Arabidopsis thaliana*, a well-characterized plant in the *Brassicaceae* family, can be used as a plant model for studying the genetics underlying seed resistance to *Aspergillus* fungal infection. Wildtype (Wt) *Arabidopsis* seeds are largely resistant to fungal infection, indicating an underlying mechanism for protection against *Aspergillus* invasion. *Arabidopsis*
has a relatively small genome (~125Mb) that was sequenced in 2000 (The Arabidopsis Genome Initiative, 2000). The plants are small at maturity and have an eight-week generation time. *Arabidopsis* flowers self-pollinate, reducing the labor requirements for plant care methods. Additionally, there are simple procedures for performing genetic crosses between *Arabidopsis* lines. Many mutant *Arabidopsis* lines are available from the Arabidopsis Biological Resource Center. *Arabidopsis*, as opposed to maize or peanut, provides a simpler system for studying plant genetics.

*Arabidopsis* seeds and *A. nidulans* fungus provide a promising two-model pathosystem. Most approaches to studying *Aspergillus* resistance in plants begin with a susceptible host and a search for resistant cultivars, then culminate in the identification of the genes involved in increased resistance (for examples, see Wilson *et al*., 2001; Pechanova *et al*., 2011; Kelley *et al*., 2012). Using *Arabidopsis*, a naturally resistant host, as a model organism allows us to take a reverse genetics approach. We can first identify genes of interest using the many web-based tools available for *Arabidopsis* genetics research (for example, The Arabidopsis Information Resource (TAIR), The Bio-Analytic Resource for Plant Biology (BAR), and Genevestigator). We can then assign susceptibility phenotypes using available mutant lines. This pathosystem allows researchers to determine if a specific gene or set of genes are necessary for conferring *Aspergillus* resistance in plants.

*Arabidopsis* seed coat (testa) development:

Angiosperm seeds are composed of the embryo, the endosperm, and the seed coat (testa). Seed development is initiated with double fertilization of the ovule by the two sperm nuclei of a pollen grain. The ovule consists of maternal integument surrounding seven meiotic cells, six haploid and one that is double-nucleated. The haploid egg cell, situated at
the micropylar end between the two synergid cells, is fertilized to form the embryo. The double nucleated cell, between the micropylar and chalazal ends of the ovule, is fertilized to form the endosperm. The endosperm releases an unknown signal that initiates differentiation of the surrounding integument layers into a mature seed coat (reviewed by Figueiredo and Kohler, 2014).

In *Arabidopsis*, the maternal integument in the ovule is made up of five layers of cells that grow via both cell division and cell growth after fertilization to form the testa. The differentiation process of the testa is complete by fifteen days after fertilization (Haughn and Chaudhury, 2005). Two cell layers make up the outer integument (the epidermal and palisade layers) and three layers comprise the inner integument (Figure 1). The cells in the epidermal layer (Figure 1) produce and secrete large amounts of polysaccharide mucilage into the space between the plasma membrane and the cell wall. This secretion of mucilage forces the cytoplasm into the center of the epidermal cells, forming columella, after which thick secondary cell walls are formed (Windsor et al., 2000; Western et al., 2000; Haughn and Chaudhury, 2005). The stored mucilage is not released from the outer epidermal layer until the mature seeds undergo imbibition at the initiation of germination (Western et al., 2000). The palisade cells (Figure 1) form thick secondary cell walls on the inner side of the cell before undergoing apoptosis (Haughn and Chaudhury, 2005). The secondary cell walls produced by the outer integument cell layers provide the embryo with a physically strong encasement even after the cells are dead and the palisade layer has collapsed. The two outer layers of the inner integument do not seem to further differentiate before they undergo programmed cell death (Nakuane et al., 2005; reviewed by Hatsugai et al., 2015; reviewed
by Van Hautegam et al., 2015), after which they collapse and are compacted together with
the palisade layer (Haughn and Chaudhury, 2005).

The cells in the innermost layer of the inner integument (the endothelium; Figure 1),
synthesize phenylpropanoid molecules that accumulate for the first week after fertilization
(Haughn and Chaudhury, 2005). Some phenylpropanoid molecules (proanthocyanidins) then
begin to oxidize, providing Arabidopsis seed coats with their characteristic brown
pigmentation (reviewed by Winkel-Shirley, 2001). At the end of seed coat maturation, the
phenylpropanoid molecules are released from the endothelial cells into the intracellular space
as the cells undergo programmed cell death (Lepiniec et al., 2006).

**Phenylpropanoids protect plants against *Aspergillus* infection:**

Phenylpropanoids are a diverse set of molecules, produced via the phenylalanine
ammonia lyase pathway, that provide seeds with protection from abiotic and biotic stressors
(reviewed by Tohge et al., 2013). Lignin molecules (polymerized phenolics) provide plants
with mechanical protection by making cell walls tougher and more water resistant, thereby
protecting against fungal penetration and against enzymes that can degrade the cell wall
(reviewed by Naoumkina et al., 2010; Kavousi et al., 2009). In maize, infection often occurs
through cracks or wounds, indicating that lignin could be involved in providing kernels with
a mechanical barrier to infection (Naoumkina et al., 2010). Lignin and its precursors may
also act chemically as pathogen inhibitors (reviewed by Naoumkina et al., 2010). However,
the involvement of lignin in inhibition of bacterial and fungal infection is disputed. For
example, King and Scott claim that kernel hardness does not correlate with maize
susceptibility to fungal infection (1982). Funnell and Pedersen showed that a reduction of
lignin content in sorghum leads to increased, rather than decreased, resistance to both bacterial and fungal infection (Funnell and Pedersen, 2006; reviewed by Naoumkina, 2010).

Phenylpropanoids likely contribute to phytoalexin chemical inhibition of fungal pathogens. Plants that are resistant to fungal infection, including resistant cultivars of maize and peanut, often show an upregulation of phenylpropanoid and flavonoid gene expression in green tissues after being challenged by fungal pathogens (reviewed by Naoumkina et al. 2010; Mierziak et al., 2014; Starr et al., 2014; Wang et al., 2016a). Stilbenes, though not present in many plants (Schröder, 1997; Dixon et al., 2002), are an important factor in peanut resistance to Aspergillus (Wotton and Strange, 1985; reviewed by Liang et al., 2006; Sobolev et al., 2007). Many of the flavonoid biosynthesis genes are upregulated during seed coat development (Winkel-Shirley, 2001; Abrahams et al., 2002; Haughn and Chaudhury, 2005; Dean et al., 2011), and we hypothesize that flavonoids contribute to innate fungal resistance in mature seeds. It would be useful to discern which product of the flavonoid pathway is essential in Aspergillus resistance. A flavonoid gene conferring resistance in Arabidopsis could be present in maize and peanut as well, since parts of the phenylalanine ammonia lyase pathway (including flavonoid biosynthesis) are highly conserved amongst both monocots and dicots (reviewed by Dixon et al., 2002; Rawal et al., 2013); however, further analysis would be necessary in order to determine if the product was produced in agriculturally important crops, and if it was actively inhibitory in those plant systems.

Previous colleagues demonstrated that mutants in chalcone synthase, the first dedicated enzyme in flavonoid biosynthesis, were infectible by Aspergillus (Brodhagen and Young, unpublished data). Flavonoids, a subset of phenylpropanoids, are derivatives of the fifteen-carbon flavan molecule (Figure 2a). They have been previously shown to chemically
inhibit Aspergillus mycelial growth (for examples, see Weidenbörner et al., 1990; Kanwal et al., 2010). Additionally, mature seeds that are resistant to fungal infection can show a higher expression rate of flavonoid biosynthesizing genes when compared to seeds that are susceptible to fungal infection. For example, flavonoid-specific enzymes such as chalcone synthase (CHS) are upregulated in mature peanut seeds that are resistant to Aspergillus fungal infection (Wang et al., 2016a). A detailed understanding of flavonoid biosynthesis will help identify the specific proteins that are involved in providing Arabidopsis seeds with resistance to Aspergillus.

**Flavonoids: biosynthesis, modification, and transport in Arabidopsis:**

The identification of mutant Arabidopsis lines with impaired flavonoid accumulation and altered seed coat pigmentation (Koornneef, 1990), named transparent testa mutants (Table 1), has helped identify many of the genes and proteins involved in the accumulation of flavonoid molecules (Shirley et al., 1995; Abrahams et al., 2002). Arabidopsis seeds accumulate at least fifty-four flavonoid molecules (reviewed by Saito et al., 2013), including flavonols, flavan-3-ols, and epicatechin-based proanthocyanidins (Figure 2b,i, ii, iii, and Figure 3) (Routaboul et al., 2006). There are four main functional categories of proteins involved in flavonoid production: enzymes involved in the biosynthesis of the flavonoid scaffolds, enzymes that modify the scaffold structures, transport proteins, and transcriptional regulatory factors (Figure 3) (reviewed by Saito et al., 2013; reviewed by Appelhagen et al., 2014; reviewed by Lepiniec et al., 2006).

In Arabidopsis, there are nine enzymes known to be involved in the biosynthesis of flavonoid scaffold molecules (Figure 3). Chalcone synthase (CHS), the first enzyme in the flavonoid branch of the phenylpropanoid biosynthetic pathway, catalyzes the condensation
reaction of \( p \)-coumaroyl CoA with three molecules of malonyl CoA to form chalcone (tetrahydroxychalcone) (Austin and Noel, 2003). After CHS initiates this first committed step in the production of flavonoids, chalcone isomerase (CHI) catalyzes the cyclization reaction of chalcone into naringenin (a flavanone, Figure 2b,iv). Naringenin is then oxygenated at the 3-position by flavanone 3-hydroxylase (F3H), to give dihydrokaempferol (dihydroflavonol) (Pelletier et al., 1996). Dihydrokaempferol can undergo a desaturation at the 2,3 position by flavonol synthase (FLS) to produce kaempferol, or it can be hydroxylated at the 3’ position by flavonoid 3’ hydroxylase (F3’H) to produce dihydroquercetin. Dihydroquercetin can also undergo a desaturation at the 2,3 position by FLS to produce quercetin, or it can undergo a reduction of the keto group to a hydroxyl group at the 4 position by dihydroflavonol reductase (DFR) to produce leucocyanidin (a flavan-3,4-diol, Figure 2b,v). The branches initiated by FLS mark the first committed steps in the production of flavonols, while the branch initiated by DFR marks the first committed steps in the production of proanthocyanidins. Full desaturation of the C ring of leucocyanidin is catalyzed by leucoanthocyanidin dioxygenase (LDOX), resulting in cyanidin. Cyanidin is then reduced to epicatechin by the anthocyanidin reductase (BAN) enzyme (Abrahams et al., 2003). The final biosynthetic enzyme, laccase-like 15 (LAC15), is a laccase-like polyphenol oxidase that is involved in the oxidative polymerization of flavonoids that yields proanthocyanidins (Pourcel et al., 2005), the majority of which are epicatechin-based in Arabidopsis (reviewed by Saito et al., 2013). One other laccase enzyme, LAC5, is upregulated during silique development (Figure 4), but it has no known involvement in flavonoid biosynthesis.
Accumulation of flavonoids in developing Arabidopsis seeds:

Flavonoids accumulate during Arabidopsis seed coat development. The main flavonol in mature Arabidopsis seed coats is quercetin-3-O-rhamnoside. It begins to accumulate in the endothelial cells about seven days after flowering and continues accumulating through the end of seed coat maturation (Routaboul et al., 2006). Epicatechin accumulates in high concentrations seven days after flowering, but decreases dramatically at the end of seed coat maturation, when it is polymerized to form proanthocyanidins. Soluble proanthocyanidins accumulate in the endothelial cells up to twelve days after flowering, after which their concentration quickly decreases, leaving very few soluble proanthocyanidins in the mature seed coat. Insoluble proanthocyanidins, on the other hand, begin to accumulate within four days after flowering, increase gradually in concentration, and are still present in high quantities in mature seed coats (Routaboul et al., 2006).

Modification of Arabidopsis flavonoids:

The flavonoid scaffold molecules are modified in different ways by several enzymes. Flavonoid glycosyltransferase (TT15) catalyzes the glycosylation of flavonoids, using both sterol glucoside and UDP-glucose as substrates (Figure 5a). Other enzymes catalyze the prenylation, methylation, and acylation of flavonoid scaffold molecules (see Figure 5b for prenyl-, methyl-, and acyl- groups) (reviewed by Saito et al., 2013). Another enzyme, glutathione S-transferase PHI 12 (GSTF12), is homologous to glutathione transferases; however, instead of catalyzing the addition of glutathione molecules (Figure 5c) onto flavonoid scaffold molecules, GSTF12 is believed to be involved in transporting flavonoids into the vacuole and potentially in protecting them from oxidation (reviewed by Zhao et al., 2010).
Transport and storage of flavonoids in Arabidopsis:

Several other proteins are involved in the transport and storage of flavonoids. During the early stages of seed coat development, proanthocyanidins are stored in the vacuoles of endothelial cells (Abrahams et al., 2003). Synthesis of proanthocyanidin precursors, however, is believed to occur in the cytosol near the endoplasmic reticulum, and these precursors must be transported into the vacuole (Debeaujon et al., 2001). The transparent testa 12 (TT12) enzyme, a multi-antimicrobial extrusion (MATE) family transporter (Debeaujon et al., 2001), localizes to the membrane of endothelial cell vacuoles. It is involved in proanthocyanidin transport (Debeaujon et al., 2001), but the intermediates transported into the vacuole by TT12 are not yet known. Another transporter necessary for proanthocyanidin synthesis in endothelial cells is autoinhibited H⁺-ATPase 10 (AHA10) (Baxter et al., 2005). AHA10 is a P-type H⁺-ATPase that could aid the TT12 enzyme in transporting proanthocyanidins, since MATE antiporters require an ion gradient across a membrane to drive their activity.

Regulation of flavonoid biosynthetic pathway genes:

The expression of genes in the flavonoid biosynthetic pathway is under the regulation of six known transcription factors. Three proteins form a transcription complex: MYB domain protein 123 (MYB123), which is a myeloblastosis (MYB) family transcription factor; a basic helix-loop-helix transcription factor (BHLH42); and transparent testa glabra 1 (TTG1), which is a WD-repeat (WDR) family protein. The MYB-bHLH-WDR complex is well conserved, and MYB123-BHLH42-TTG1 regulates the transcription of the genes involved in the initiation of proanthocyanidin production (Debeaujon et al., 2003). A WRKY transcription factor (WRKY44) is thought to function downstream of TTG1 (reviewed by
Debeaujon et al., 2003; reviewed by Xu et al., 2015. Arabidopsis B-sister protein (ABS) is a MADS-domain (MCM1, Agamous 1, Deficiens, and Serum response factor) protein (Nesi et al., 2002) involved in the initiation of the development of endothelial cells and their accumulation of proanthocyanidins in part by the regulation of anthocyanidin reductase (BAN) (Dean et al., 2011). WIP domain protein 1 (WIP1), has a zinc finger domain and is also involved in the regulation of anthocyanidin reductase (BAN).

**Summary:**

With such a detailed understanding of flavonoid biosynthesis in Arabidopsis, and with mutant lines available for each known gene in the pathway, we aim to decipher which flavonoid biosynthetic, transport, and regulatory genes are directly involved in providing Wt Arabidopsis seeds with resistance to Aspergillus fungal infection. This thesis will cover a dissection of the Arabidopsis flavonoid biosynthetic pathway. Since Arabidopsis does not produce anthocyanins or stilbenes, this thesis only covers the resistance mechanisms provided by a reduced set of phenylpropanoid molecules. This thesis will also take a detailed look into the variability in susceptibility phenotypes of Arabidopsis seed mutants, and will assess the robustness of the Arabidopsis and A. nidulans pathosystem. This work could have implications for understanding and improving crop seed resistance to A. flavus infection and aflatoxin contamination.
METHODS

**Plant care for Arabidopsis thaliana:**

*Arabidopsis thaliana* seeds of the Columbia and Landsberg ecotypes were received from the Arabidopsis Biological Resource Center (Ohio State University). Strains included wildtype Columbia (Wt Col) and wildtype Landsberg (Wt Ler), as well as strains with single gene mutations in flavonoid biosynthetic pathway genes (*chs, fls, f3’h, dfr, ldox, ban, lac15, tt15, gstf12, tt12, aha10, bhlh42, ttg1, and wip1*), in an additional laccase gene (*lac5*), and in the *ERECTA* gene (*er*) (Table 1). The seeds were vernalized at 4°C for seven days. They were then surface sterilized in 70% EtOH/0.01% Triton X-100 for five minutes, followed by a 95% EtOH rinse for one minute. The seeds were planted on 0.5X Murashige Skoog (MS) 1% agar media and placed at 4°C in the dark for three days, before being placed at 21°C under fluorescent light for 14 days. The seedlings were transplanted into a 2:1:1 sterilized mixture of SUNSHINE® Professional Growing Mix #2 media (SUN GRO Horticulture Canada Ltd, Seba Beach, AB, Canada), Plant!t Super Coarse Perlite (Hydrofarm, Petaluma, CA), and Hoffman® Horticultural Vermiculite (Good Earth™, Lancaster, NY) using Miracle Gro® fertilizer (0.04g/L DI H2O; 30% N, 10% P, 10% K, less than 1% B, Cu, Fe, Mn, Mo, Zn; product #1001791) (Scotts Miracle-Gro Products, Inc., Marysville, OH). The plants were grown at 21°C under fluorescent light (F4012/C50 SUPREME; 40 watts; Alto Technologies, Phillips Lighting Company, Somerset, NJ) with a sixteen-hour day, and were watered with Miracle Gro fertilizer (0.08g/L DI H2O) when the pots were no longer heavy with water. Cohorts of seeds grown over two years ago were grown under a 24-hour light regimen.

**Whole-plant-harvesting:** Six weeks after transplanting, the plants were left unwatered and allowed to dry for two to eight months at 21°C under fluorescent light. Seeds were
whole-plant harvested and stored in microcentrifuge tubes at room temperature. Each microcentrifuge tube held seeds from one parent plant, with a range in seed ages spanning up to seven weeks difference (Figure 6). It is of note that plants in the Ler genetic background often have less biomass than plants in the Col background. They are often slower to begin development, but faster to complete it, than Col ecotype plants. Under our experimental conditions, plants from different ecotypes were not segregated into separate watering trays, so the smaller Ler ecotype plants were likely overwatered.

**Fungal care for Aspergillus nidulans:**

Wildtype *Aspergillus nidulans* RDIT 9.32 was obtained from Dr. Nancy P. Keller, and stored at -80°C in glycerol. A serial dilution of *A. nidulans* was plated out on 1.6% (w/v) agar Glucose Minimal Media (GMM) (0.056 M glucose, 0.07 M NaNO₃, 7E-3 M KCl, 2E-3 M MgSO₄•7H₂O, 0.011 M KH₂PO₄, 7.6E-5 M ZnSO₄•7H₂O, 1.8E-4 M H₃BO₃, 2.5E-5 M MnCl₂•4H₂O, 1.8E-5 M FeSO₄•7H₂O, 7.3E-6 M CoCl₂•5H₂O, 6.4E-6 M CuSO₄•5H₂O, 1.7E-7 M (NH₄)6Mo₇O₂₄•4H₂O, 1.3E-4 M Na₂EDTA; pH 6.5; 16 g agar in 1 L) (Bailes *et al.*, 2013) and incubated at 37°C in the dark. After 24-36 hours of growth, new GMM plates were point inoculated from single germinated spores and incubated at 37°C under broad-spectrum light (Halco Lighting Technologies, Norcross, GA). After seven days, the plates were stored at 4°C with Parafilm. From these stock cultures, *A. nidulans* was streaked on GMM agar and incubated for 48 hours at 37°C under broad-spectrum light. The spores were harvested in 0.01% (v/v) Triton X-100, stored at 4°C in a 15 mL conical vial, and used in bioassays within 12 hours.

**Assessment of Arabidopsis seed susceptibility to A. nidulans:**

Humidity chambers were aseptically prepared in NUNC® six-well plates (Thermo
Fisher Scientific, Roskilde, Denmark). Two pieces of #1 grade, 2.5 cm diameter Whatman™ filter paper (GE Healthcare Life Sciences, Little Chalfont, Buckinghamshire, UK) in each well were wetted with 100 µL of sterile Nanopure H₂O. To help maintain high humidity, a microcentrifuge tube cap (removed from a 1.5 mL tube; Thermo Scientific, San Diego, CA) in each well was filled with 230 µL of sterile Nanopure H₂O. Approximately 90 seeds by volume (plus or minus 10 seeds) were placed in microcentrifuge tubes for wetting. 250 µL of 0.01% Triton X-100 was added to each microcentrifuge tube before seeds were pipetted onto the filter paper of their corresponding wells in 150 µL of 0.01% Triton X-100. The seeds in each well were inoculated with 10⁶ wildtype A. nidulans RDIT 9.32 spores in 15 µL of 0.01% Triton X-100, and incubated at 37°C in the dark. After an initial 16 hours, and then at 12 hour intervals, the filter papers in the four corner wells were rehydrated with 80 µL of sterile Nanopure H₂O and the two center wells with 60 µL. After 48 to 72 hours, or until sporulation occurred on the positive control seeds (old chs seeds from the tt4 line in the Ler background), the top pieces of filter paper, along with the inoculated seeds, were placed in individual 50 mL Falcon® tubes (Corning Science, Reynosa, Tamaulipas, México) in 1 mL of sterile 0.01% Triton X-100. The tubes were vortexed for one minute; then, from the average of four counts on a hemocytometer, the average concentration of spores per seed was calculated for each replicate sample. A minimum of three biological replicates was run per seed type. The infection rates of individual mutant seed strains were compared to the infection rate of the wildtype seeds using an unpaired two-tailed t-test, with an alpha value of 0.05. Each experiment was repeated at least once.

Two controls were run with each experiment. First, there was a no-spore trial for each seed type to control for the fact that seeds were not surface-sterilized prior to the seed
infection assays; this control ensured that native epiphytic fungi were not present and were not contributing to the observed fungal growth. Each seed type had one well with 15 µL of 0.01% Triton X-100, instead of inoculation with 15 µL of fungal spore suspension (results not shown). Second, there was a no-seed control to ensure that the fungus was viable. Filter paper without any seeds was inoculated with $10^6$ A. nidulans spores, in a minimum of three biological replicates, and quantification of fungal spores was calculated as described above.

**Timecourse of Arabidopsis seed infectibility:**

Buds of *Arabidopsis thaliana* Wt Col and *chs* plants were marked weekly, to yield cohorts of seeds harvested at similar days after flowering (DAF), with a resolution of one week (Figure 6). Three new seedlings from each genotype were planted every two weeks. The plants were watered with Miracle Gro fertilizer (0.08g/L DI H$_2$O) when the surface of the planting media started to dry.

**Silique-harvesting:** One week after peduncle collapse, seeds were harvested siliquenby-siliquen and stored in microcentrifuge tubes at room temperature. Each microcentrifuge tube held seeds that were within a week of the same age, and which came from an average of nine different plants. Cohorts were marked for a ten-month period. Infectibility of older and younger seeds was compared, using the protocol for the assessment of seed infectibility described above.

**Analysis of Arabidopsis seed germination rates:**

Seeds were sterilized as described above in Plant Care. Twenty to sixty seeds were then planted in triplicate on 0.5X Murashige Skoog (MS) 1% agar media in 100 X 15mm Petri plates using a pick sterilized with 70% ethanol. They were held at 4°C in the dark for
three days, before being placed at 21°C under fluorescent light. The percentage of germinated seeds per plate was quantified after ten days.

**Analysis of the inhibitory properties of aqueous extracts from Arabidopsis seeds:**

Aqueous seed extract was prepared with 0.1 mL (by volume) of each strain of Arabidopsis thaliana seeds. Seeds were soaked in 600 µL of sterile ddH₂O in microcentrifuge tubes for fifteen minutes with shaking at 30 rpm, followed by 15 seconds of vortexing. The seeds were ground using one hundred quick pulses from a microcentrifuge bit attached to a VirTis “45” drill (Gardiner, NY) on low. An additional 800 µL of DI H₂O was added to each tube, followed by 15 seconds of vortexing. Tubes were centrifuged at maximum speed (14000 rpm) for three minutes, after which the aqueous extract supernatant was pipetted into a sterile microcentrifuge tube. An agar base of 1 mL solid Glucose Minimal Media (1.6% agar w/v) was pipetted into capless 1.5 mL microcentrifuge tubes (Thermo Scientific, San Diego, CA), and allowed to solidify. A soft agar overlay was prepared with 20 µL of aqueous extract added to 130 µL of semisolid Glucose Minimal Media (0.8% agar w/v) containing a suspension of 10⁴ spores/mL of A. nidulans RDIT 9.32. The solution was vortexed for 30 seconds on maximum speed, after which 150 µL was spread evenly over the solid GMM base, into the respective capless microcentrifuge tubes. Another capless, sterile microcentrifuge tube was secured over each experimental tube, using micropore tape. The tubes were incubated agar-side-up at 37°C in the dark for 45 hours. The contents of each tube were added to 3 mL of 0.01% Triton X-100, then crushed with a pestle to a uniform consistency and vortexed for 1 minute at top speed. A hemocytometer was used to enumerate spores/cm². Each treatment (seed extract from a particular genotype) consisted of five biological replicates. Each experiment was repeated a minimum of two times. Two controls
were run with each experiment: sterile Nanopure H₂O instead of aqueous seed extract, and 0.01% Triton X-100 instead of spore suspension.
RESULTS

Validation of the pathosystem:

Wildtype Col and Ler Arabidopsis thaliana seeds are both resistant to Aspergillus nidulans infection (Figure 7a). Arabidopsis seeds mutant in chalcone synthase (chs) are susceptible to both A. flavus (Figure 7b) and A. nidulans (Figure 7c), with infection often occurring at the micropylar end of the seeds (data not shown). The susceptible chs seeds are in the Landsberg genetic background and have a second mutation in the ERECTA gene. However, a single er mutation does not diminish the resistance of Col or Ler Arabidopsis seeds to A. nidulans fungus (Figure 7d).

A genetic dissection of the flavonoid biosynthetic pathway, to identify Aspergillus resistance factors:

A genetic dissection of the flavonoid biosynthetic pathway in Arabidopsis revealed that mutations in chs, f3’h, and dfir result in seeds that are more susceptible than Wt Col seeds to A. nidulans (Figure 8a, b, c; Figure 3), while seeds with mutations in fls, ldox, and ban remain resistant to infection (Figure 8d, e, f; Figure 3). Mutations in the known modifying enzymes (tt15 and gsf12) and the known transporters (tt12 and aha10) that are involved in flavonoid production do not result in susceptibility to A. nidulans (Figure 8g, h, i, j; Figure 3). A mutation in the wip1 transcription factor results in susceptibility, while a mutation in the bhhlh42 transcription factor does not (Figure 8k, l; Figure 3). A mutation in lac15, a laccase-like enzyme known to act in the flavonoid biosynthetic pathway, results in seeds that are mildly susceptible to A. nidulans infection (Figure 8m, Figure 3; Figure 9). Seeds with a mutation in lac5, a laccase enzyme that is upregulated during silique development, but has no known involvement in flavonoid biosynthesis, remain resistant to A. nidulans (Figure 9). It is
of note that the seed infectibility assays described here were performed with seeds that were harvested from entire plants and allowed to mature to at least 133 days after flowering.

**Timecourse of Arabidopsis seed infectibility:**

We noted that older whole-plant-harvested chs mutant seeds were sometimes more susceptible to *A. nidulans*, while younger mature seeds are less susceptible (example, Figure 10). Therefore, a timecourse assay was designed to measure seed susceptibility over time, comparing Wt Col and chs seeds from plants whose siliques were marked at one-week intervals and individually harvested one week after peduncle collapse. Developing seeds from both Wt Col and chs plant lines are susceptible to infection, but resistance develops in both seed types when they reach maturity between 8 to 18 days after flowering (Figure 11). In contrast to our observations from whole-plant-harvested seeds, silique-harvested seeds remained resistant to *A. nidulans* regardless of age (Figure 11). The timecourse was only performed once, but its results were so unexpected as to prompt us to examine possible developmental differences between whole-plant- and silique-harvested seeds.

**Effect of seed development on chs seed susceptibility to A. nidulans infection:**

Ten months after flowering, silique-harvested chs seeds were not susceptible to *A. nidulans* (Figure 12b). However, ten-month-old chs seeds that were whole-plant-harvested after an eight-month drying period were highly susceptible (Figure 12b). The silique-harvested chs seeds are a vibrant, yellow color (Figure 12a), and have a germination rate over 99% (Figure 12c). The whole-plant-harvested chs seeds are a dull, grey-yellow color (Figure 12a), and have a germination rate of about 40% (Figure 12c). While the silique-harvested seeds remain yellow during infection experiments, most of the whole-plant-harvested chs seeds visibly turn darker after 32 hours at 37°C in the dark (data not shown).
The seeds that turn darker during incubation will not germinate, even if they are placed under fluorescent light at 21°C (data not shown).

Closer inspection revealed that some cohorts of *chs* seeds have a mixture of phenotypes: larger, yellow seeds vs. smaller, darker seeds (Figure 13a). The smaller, darker *chs* seeds are those that are produced in the late-forming siliques that often develop in tight clusters after the plant has been taken off of water (Figure 6, week 7). These seeds are an average of 44% smaller than seeds produced in siliques from the main branches of an *Arabidopsis* plant (Figure 13b). The yellow seeds are resistant to *A. nidulans*, while isolated late-forming seeds are highly susceptible to infection (Figure 13c). When late-forming *chs* seeds were mixed with either Wt Col seeds or with yellow *chs* seeds, inoculation with *A. nidulans* spores resulted in high infection of the late-forming seeds by 36 hours while at 48 hours all other seeds were clear of infection (data not shown). The yellow seeds have a germination rate of 97%, while the small, late-forming seeds have a reduced germination rate of only 15% (Figure 13d), and the resulting seedlings are stunted in growth (Figure 13e).

**Effect of genotype on *chs* seed susceptibility to *A. nidulans* infection:**

When four different *chs* mutant seed types were inoculated with *A. nidulans*, they showed variability in susceptibility, correlating with their genetic background (Figure 14a). Seeds with mutations in the Ler genetic background (*tt4* and *tt4-I*) were more highly infected than those in the Col genetic background (*tt4-3* and *tt4-TDNA*). All seeds were used 70 days after flowering, and all parent plants were from the same cohort and were planted, watered, and whole-plant-harvested on the same dates; plants from different ecotypes were not segregated into separate watering trays. A germination test of these *chs* seeds showed that the
two mutant seed types that had a higher susceptibility to infection also had lower germination rates (Figure 14b).

**Mode of wildtype Arabidopsis resistance to Aspergillus:**

The aqueous extract from Wt Col seeds prevents *A. nidulans* growth, while the fungus grows and sporulates prolifically in a no-extract water control (Figure 15). However, aqueous extracts from *chs* seeds variably affect fungal growth, even when all seeds have the same allelic mutation and are grown and stored under the same temperatures and light frequencies. The effect of *chs* seed extracts on *A. nidulans* does not correlate with the infectibility, germination rates, or age of the seeds (Figure 16).
DISCUSSION

**Creation of a two-model pathosystem:**

Resistance of wildtype *Arabidopsis thaliana* seeds to *Aspergillus* fungi indicates the presence of a mechanical and/or chemical barrier to infection. The identification of mutant *Arabidopsis* seeds that are susceptible to *Aspergillus nidulans* provides a two-model pathosystem for studying the mode of seed resistance to *Aspergillus*. Mutant *Arabidopsis* seeds in either the Col or the Ler genetic background can be used in this pathosystem to find genes that are necessary for fungal resistance.

**Flavonoids involved in *Arabidopsis* seed resistance to *A. nidulans:***

Susceptibility of seeds mutant in the first enzyme of the flavonoid biosynthetic pathway, chalcone synthase (*chs*), suggests that a downstream molecular product(s) could be responsible for conferring resistance in wildtype *Arabidopsis* seeds. The genetic dissection of the flavonoid biosynthetic pathway shows that genes from *CHS* through *DFR* are necessary for conferring resistance to *Aspergillus*, while genes at the heads of the flavonol and proanthocyanidin branches (*FLS* and *LDOX*, respectively) may not be necessary. This would suggest that leucocyanidin, the molecular product of the last necessary gene (*DFR*), is required for *Arabidopsis* seed resistance to *Aspergillus*. It also suggests that desaturation of the C ring is not necessary for fungistatic activity of the active compound. We have not yet tested whether leucocyanidin is directly or indirectly inhibitory to *Aspergillus*, since our attempt to synthesize the molecule via a sodium borohydride (NaBH₄) reduction of dihydroquercetin was unsuccessful. Future research should include an analysis of the inhibitory properties of the leucocyanidin molecule, to determine if it has direct fungicidal
properties against *Aspergillus*, or if leucocyanidin acts as a precursor for the active molecule(s) that provides wildtype *Arabidopsis* seeds with resistance.

If leucocyanidin is not directly active, susceptibility phenotypes of *Arabidopsis* seeds with mutations in known flavonoid modifying enzymes, transporters, and laccases provides insight into possible conversion steps of leucocyanidin from an inactive to an active molecule. Since *tt15, gstf12, tt12, and aha10* mutant seeds are resistant to *A. nidulans* infection, conversion of leucocyanidin would not likely rely on glycosylation by TT15, glutathione transferase activity or transportation by GSTF12, or transportation into vacuoles by TT12 or AHA10. The susceptibility of *lac15* mutant seeds to *A. nidulans*, however, suggests that the laccase is involved in providing seeds with *Aspergillus* resistance. LAC15 acts on several substrates and has known activity in the production of biflavonols and oxidized proanthocyanidins (Figure 3). However, since *fls* and *ldox* mutant seeds are resistant to *A. nidulans*, those branches of the pathway are not necessary for resistance. To create a chemical resistance factor, LAC15 could either act on an unknown substrate, or, in a novel branch of the flavonoid biosynthetic pathway, LAC15 may use leucocyanidin as a precursor in producing an active anti-fungal molecule (Figure 3). Since *lac15* seeds are only mildly susceptible to *A. nidulans*, its activity can be assumed necessary, but not sufficient, for resistance; i.e., another factor(s) is necessary to achieve wildtype levels of resistance. A reasonable hypothesis is that another laccase is functionally redundant to LAC15. Though the biological role of LAC5 has not yet been identified, it is the only other laccase in *Arabidopsis* that is highly upregulated in mature siliques (Figure 4). Though a single mutation in *LAC5* is not sufficient to cause susceptibility, a *lac15/lac5* double mutation may be more susceptible.
than either single mutant. Further investigation is necessary for determining the involvement of laccases in conferring resistance against *Aspergillus*.

**Resistance of developing vs. mature *Arabidopsis* seeds:**

Susceptibility of both Wt Col and *chs* seeds varies over time. Developing seeds from both plant lines are highly susceptible to *A. nidulans* until they reach maturity, at which point they both develop resistance. This susceptibility of young seeds, which to our knowledge has not yet been described, suggests that green seeds may rely on the parent plant for protection until they produce a mechanical and/or chemical barrier to infection (for a detailed review in seed development, see Chaudhury *et al.*, 2001; Lepiniec *et al.*, 2006; Bentsink and Koornneef, 2008; Le *et al.*, 2010). Mature Wt Col seeds remain resistant to *A. nidulans*, indicating that the barrier(s) are maintained over time. Mature *chs* seeds, however, show variability, sometimes remaining resistant to *A. nidulans* over time, but sometimes developing susceptibility. This indicates that a barrier in *chs* seeds may be inconsistently lost or broken down over time.

**Variability in *chs* seed infection correlates with variation in germination rates:**

The variability in susceptibility of *chs* seeds to *A. nidulans* correlates with seed viability: higher rates of infection correlate with lower germination rates. This indicates that *A. nidulans* infects dead or weak seeds more readily than it infects living seeds, as is expected of a saprophytic fungus. These differences in seed susceptibility and viability appear under several changes of variables.

*Growth conditions:*

There are differences in seed viability amongst seeds grown in different conditions. A longer drying period for the parent plant before seed harvesting appears to result in reduced
viability of seeds, suggesting that long lengths of time under fluorescent light reduces fitness in chs seeds, but not in Wt Col seeds. Seed viability could be reduced by either ultraviolet light damage or via oxidative stress. The flavonoid biosynthetic pathway, rendered non-functioning in chs seeds, is important for protection of plant tissues against ultraviolet light damage (Li et al., 1993; Landry et al., 1995). Flavonoids absorb ultraviolet light, thereby preventing direct damage to DNA and RNA. They also function as antioxidants, protecting the cells from reactive oxygen species (reviewed by Agati and Tattini, 2010), which increase under ultraviolet light stress (Rozema et al., 1997). Additionally, in green tissues, oxidative damage to membranes caused by heat stress is induced by light exposure (Larkindale and Knight, 2002), indicating that seeds not protected by flavonoids could be more vulnerable to oxidative damage. Since fluorescent lights can release ultraviolet light wavelengths (Mironava et al., 2012), it is plausible that excessive light exposure under our experimental conditions damages chs seeds more readily than wildtype seeds (for examples, see Shaukat et al., 2013; Sullivan et al., 2014; Wang et al., 2016b), thereby causing lower rates of germination for chs, but not Wt Col seeds, from cohorts with long drying periods prior to harvest.

Water stress of the parent plant may also play a role in seed fitness. Under conditions of excess water during seed development, seeds from many plant species decrease in impermeability, and therefore increase in leakiness (reviewed by Jaganathan, 2016). Our watering regimen for whole-plant-harvested seeds was inconsistent from cohort to cohort, producing variability in levels of water stress and excess for the parent plants, and likely contributed to variability in seed desiccation and viability. The parent plants of siliquie-harvested seeds, however, were watered at optimal intervals, which may contribute to the
uniformity seen in their viability. Additionally, wildtype seeds have flavonoid proanthocyanidin polymers to help prevent leakage of solutes, but chs seeds have thinner and more permeable seed coats, and thus weaken at a faster rate (Debeaujon et al., 2000). This could result in increased chs seed desiccation over time, contributing to the lower rates of germination and higher rates of infection seen in some chs seeds compared to Wt Col seeds.

Our pathosystem is prone to variability in changing environmental conditions. Future research should only use Arabidopsis seeds that are harvested immediately after drying and which are watered at optimal intervals.

*Parent plant’s developmental stage when seeds were produced:*

The time at which a chs seed is produced during the parent plant’s development seems to affect seed viability, with late-forming seeds showing reduced rates of germination and growth. The late-forming chs seeds develop after the watering period has ended and the parent plant has begun to desiccate. This results in drought conditions during development, which is known to shorten the seed-filling period and reduce the availability for nutrient transfer from the parent plant into the developing embryo (Seiler et al., 2011; reviewed by Mitchell et al., 2016). Additionally, the location of seeds on a parent plant can greatly influence the availability of nutrients for developing seeds, resulting in varied seed fitness and a wide-range of germination rates of seeds from a single plant (reviewed by Mitchell et al., 2016). Due to a reduction in both water and nutrient availability, it is possible that the late-forming chs seeds produce fewer chemical barriers to infection and/or have reduced seed coat integrity when compared to the chs seeds produced at the peak development of the parent plant, explaining in part why A. nidulans more readily infects late-forming seeds.

Analysis of flavonoid gene involvement in seed susceptibility would be more accurate if late-
forming seeds were excluded from the bioassays. This would help ensure that susceptibility phenotypes are not skewed by the varied abundance of late-forming, underdeveloped seed outliers.

**Genetic backgrounds:**

Seeds with different genetic backgrounds can show variability in seed viability. This variability amongst seeds grown in the same conditions, (with identical planting, transplanting, and harvesting dates) suggests that the genetic background of seed lines or the presence of a second mutation may influence the rate at which chs seeds die.

It appears that chs seeds in the Ler background have higher rates of infection and lower rates of germination than chs seeds in the Col background (Figure 14a). Wildtype Col and Ler seeds, however, are genetically very similar. They originated from the same non-homozygous population of Arabidopsis plants, collected near Limberg, Germany. This would suggest that the differences seen in seed viability are not dependent on the genetic background, but may be due to the presence of a second mutation.

Irradiation of some seeds from the non-homozygous Limberg population created the erecta (er) mutation, and Landsberg erecta (Ler) was used as the genetic background for several subsequent mutant Arabidopsis lines, including each susceptible Arabidopsis line from chs through dfir, and lac15. Though the er gene mutation in Landsberg genetic background mutants is not sufficient for seed susceptibility to Aspergillus (Figure 7d), an er mutation in green tissues is known to increase plant susceptibility to pathogens such as the bacterium Ralstonia solanacearum, the necrotrophic fungus Plectosphaerella cucumerina, and the oomycete Pythium irregulare (Godiard et al., 2003; Llorente et al., 2005; Adie et al., 2007; Sánchez-Rodríguez et al., 2009; reviewed by Miedes et al., 2014). Susceptibility of
green tissues of *er* mutant plants appears to correlate with an altered cell wall composition (Sánchez-Rodríguez et al., 2009; reviewed by Miedes et al., 2014). It is formally possible that the *er* mutation in Ler genetic background mutants decreases seed viability over time by further altering the composition of the seed coat cell walls during development. The *er* mutation could therefore augment the infectibility phenotype of flavonoid biosynthetic pathway mutants. Furthermore, the *er* mutation results in plants that are shorter, and tend to have a decreased biomass. Under our experimental conditions, with plants of all ecotypes sharing watering trays, smaller plants tended to get overwatered. Excess water could contribute to an increased permeability (reviewed by Jaganathan, 2016) of *chs* seeds in the Ler genetic background. Further investigation is needed in order to determine if the *er* mutation effects the susceptibility phenotypes and viability of other flavonoid mutations. *Arabidopsis* seeds may have both mechanical and chemical barriers to *A. nidulans* infection:

Since all wildtype seeds and some *chs* seeds are resistant to *A. nidulans*, we hypothesize that a mechanical barrier to *Aspergillus* infection, consisting of the outer cell layers of the seed coat along with the micropylar plug, is initially present in *Arabidopsis* seeds. Environmental differences that result from varying plant care methods, such as light damage and excessive watering during seed development, can result in seeds that do not develop as strong of a mechanical barrier during development and are therefore more susceptible to embryonic damage and desiccation (Debeaujon et al., 2000; reviewed by Mitchell et al., 2016). These stressors put on the parent plant could thereby contribute to decreased germination rates and increased susceptibility to *Aspergillus* infection. More
research is required in order to fully understand why the mechanical barriers of some seeds are weaker than others.

Our data suggest that further resistance of *Arabidopsis* seeds is provided by water-soluble, chemical resistance factor(s) that prevent *A. nidulans* proliferation in the absence of mechanical barriers. The aqueous seed extract from Wt Col seeds reduces the germination rates of *A. nidulans* spores (Brodhagen, unpublished data), indicating that the chemical resistance factor(s) are a growth retardant, but are not fungicidal. The chemical barrier(s) are consistently present in Wt Col seeds, but variably so in *chs* seeds (Figure 16c). Although abiotic and biotic stressors can result in fluctuations in flavonoid biosynthesizing genes (for examples, see Shaukat et al., 2013; Wang et al., 2016a; Wang et al., 2016b), flavonoids are produced at basal levels in *Arabidopsis* under normal growth conditions (reviewed by Saito et al., 2013). This indicates that a flavonoid inhibitory compound would always be present, to some degree, in wildtype seeds. However, *chs* seeds, which lack flavonoids, can also inhibit fungal growth. This supports that there are both flavonoid and non-flavonoid inhibitory, water-soluble resistance factors present in *Arabidopsis* seeds. The genes responsible for producing non-flavonoid inhibitory compound(s) may only be turned on under specific growth conditions or stressors during the development of the plant, explaining in part the variability seen in *chs* seed susceptibility; however, further investigation is required before definitive conclusions can be drawn.

One remaining confounding factor is that we have not yet formally ruled out the potential effects of fungal growth enhancer(s) such as pectins, celluloses, and lipids (reviewed by Bennett, 2010). Mucilage, for example, is a water-soluble polysaccharide secreted after seed imbibition, composed primarily of pectin in *Arabidopsis* (Goto, 1985).
Though preliminary data indicates that mucilage does not affect fungal growth and sporulation (Young, unpublished data), analysis of *Arabidopsis* seeds mutant in mucilage production genes (reviewed by Moïse *et al*., 2005) would help confirm these results. Some positive enhancers, such as oils, have an increased concentration in flavonoid-deficient *Brassicaceae* species (Simbaya *et al*., 1995), and can fluctuate with variation in environmental conditions. For example, increased light can result in an increased lipid concentration in *Arabidopsis* seeds (Li *et al*., 2006). It is possible that variability in *A. nidulans* growth is due to fluctuations in both fungal growth enhancers and inhibitors. Future research should aim to identify the role that fungal growth enhancers play in *Aspergillus* growth in the presence of *Arabidopsis* seed extracts. For example, bioinformatics tools such as Genevestigator could help identify genes that are upregulated during seed development, and which are also involved in pectin, cellulose, or lipid production. Seeds mutant in these genes could be obtained from the Arabidopsis Biological Resource Center, and could be crossed with flavonoid mutants. Double mutant susceptibility phenotypes could be compared to the susceptibility of single mutants. This assessment would enhance our *Arabidopsis* and *A. nidulans* pathosystem.
CONCLUSION

Wildtype *Arabidopsis* seeds are resistant to *A. nidulans* infection via mechanical and water-soluble chemical barriers. Pathogen resistance is typically stacked so it is not surprising to find multiple antifungal mechanisms (reviewed by Palma *et al.*, 2009; Bednarek and Schulze-Lefert, 2009; and Fountain *et al.*, 2015). In *Arabidopsis*, it is possible that a mechanical barrier may be responsible for protecting young mature seeds, while the flavonoid biosynthetic pathway may be responsible for protecting older mature seeds whose mechanical barriers have lost integrity. However, we cannot confidently assign specific flavonoid resistance factors until our pathosystem has been optimized.

The pathosystem we developed is prone to variability. Our results suggest that *chs* mutant seeds are more prone to differences in development due to age and maternal effects than wildtype seeds, which is consistent with previous research (Debeaujon *et al.*, 2000). Under our experimental conditions, susceptibility of seeds was inversely correlated with germination rates. Therefore, our methods must be optimized to separate the effects of *chs* seed viability from fungal susceptibility, before a confident assignment of the flavonoid fungal resistance factor(s) can reliably be assigned. All developing *Arabidopsis* plants should be watered at optimal intervals, and should be separated by ecotype and accession to prevent overwatering of smaller plants; all seeds should be harvested immediately after the siliques begin to dry out. Additionally, under our experimental conditions, seeds in the Landsberg *erecta* genetic background were more susceptible to *A. nidulans* than seeds in the Col background. Our pathosystem must be further analyzed to determine if a second mutation in the *ERECTA* gene will consistently increase susceptibility of flavonoid mutant seeds.

Ultimately, the assessment of the chemical inhibitory properties of *Arabidopsis* seeds mutant
in different flavonoid pathway genes could help identify flavonoid molecules that actively inhibit *Aspergillus* fungus. This knowledge could aid in understanding the mechanisms underlying seed resistance to *Aspergillus* in agriculturally important crops.
REFERENCES


Figure 1. The five cell layers of a developing *Arabidopsis thaliana* seed coat, five days after fertilization. Two layers make up the outer integument (the epidermal and palisade layers), and three cell layers make up the inner integument (the endothelium being the innermost layer) (adapted from Haughn and Chaudhury, 2005).
Figure 2. The flavan backbone and classes of flavonoids. **a)** Flavan backbone: flavonoids are derivatives of the fifteen carbon flavan molecule, which has two phenyl rings (A and B) and one heterocyclic ring with an oxygen at the 1 position (C). **b)** Classes of flavonoids: 

i. **Flavonols**: 3-hydroxy derivatives of flavan with a 2,3-double bond and a 4-carbonyl group. 
ii. **Flavan-3-ols**: 3-hydroxy derivatives of flavan. 
iii. **Proanthocyanidins**: 4,8 polymerized flavan derivatives. 
iv. **Flavanones**: 4-carbonyl derivatives of flavan. 
v. **Flavan-3,4-diols**: 3,4-hydroxy derivatives of flavan.
Table 1. *Arabidopsis* flavonoid biosynthetic pathway proteins, their genes and corresponding mutant seed lines, and the mutagen used to create each mutant line. Both the conventional and the transparent testa gene names are provided. All wildtype and mutant plant lines were acquired from the Arabidopsis Biological Resource Center. Columbia (Col) genetic background (blue); Landsberg erecta (Ler) background (green); Wassilewskija background (purple). None = No mutant line used. EMS = ethylmethane sulfonate; NMU = nitrosomethyl urea; ZFN = zinc finger nuclease.

<table>
<thead>
<tr>
<th>Protein Name</th>
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<td>chs; tt4† (actually alb2•tt4-1)</td>
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<tr>
<td></td>
<td></td>
<td>tt4-TDNA*</td>
<td>T-DNA</td>
</tr>
<tr>
<td>Chalcone Isomerase</td>
<td>CHI; TT5</td>
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<td>None</td>
</tr>
<tr>
<td>Flavone-3-Hydroxylase</td>
<td>F3H; TT6</td>
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<td>Flavonol Synthase</td>
<td>FLS</td>
<td>f3′h; f3′h† (actually alb2•tt7-1)</td>
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</tr>
<tr>
<td>Flavone-3′-Hydroxylase</td>
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<td>dfr; dfr†</td>
<td>X-rays</td>
</tr>
<tr>
<td>Dihydroflavanol Reductase</td>
<td>DFR; TT3</td>
<td>tt7†</td>
<td>T-DNA</td>
</tr>
<tr>
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<td>T-DNA</td>
</tr>
<tr>
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<td>ban; ban†</td>
<td>T-DNA</td>
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<td>LAC15; TT10</td>
<td>lac15; tt10†</td>
<td>EMS</td>
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<tr>
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<td>tt15; tt15†</td>
<td>T-DNA</td>
</tr>
<tr>
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<td>gsf12; tt19†</td>
<td>T-DNA</td>
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<tr>
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<td>X-rays</td>
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<td></td>
<td>lac5-2†</td>
<td>T-DNA</td>
</tr>
<tr>
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<td>er; Ler†</td>
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</tr>
<tr>
<td></td>
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<td>er-1†</td>
<td>T-DNA</td>
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</tbody>
</table>

† Homozygous mutant seed line
* Heterozygous mutant seed line
Figure 3 (previous page). The flavonoid biosynthetic pathway in *Arabidopsis* (Figure adapted from Winkel-Shirley, 2001; Routaboul et al, 2006). Molecular changes are highlighted red within each molecular structure. Mutant seed lines are indicated in parentheses below each protein name. Mutant lines whose seeds are infected by *A. nidulans* (*red*); resistant seed lines (*blue*); untested seed lines (*black*). A proposed novel branch of the pathway is indicated in grey.
Figure 4. Levels of expression of the seventeen laccase genes in *Arabidopsis* (Hruz *et al.*, 2008). Laccase-like 15 (*LAC15*) and laccase 5 (*LAC5*) genes increase in expression at the end of seed maturation, and have higher expression rates in mature siliques than other known laccase enzymes. *LAC15* is involved in flavonoid biosynthesis, but the function of *LAC5* is not yet known.
Figure 5. Molecular changes made by flavonoid modifying enzymes. a) Substrates for flavonoid glycosyltransferase (TT15). b) Molecular groups added on to flavonoid scaffold molecules. c) Substrate for glutathione S-transferase PHI 12 (GSTF12).
Figure 6. Ages of siliques and seeds along the branches of an *Arabidopsis thaliana* plant. Black numbers represent the week after transplanting in which that silique began to develop. When seeds are whole-plant-harvested, all seeds from the entire parent plant are mixed together, and seed ages span a range of about a seven-week difference. When seeds are silique-harvested, the age range of seeds in each collection tube spans only a single week.
Figure 7. A two-model pathosystem: *Arabidopsis* seeds and *Aspergillus nidulans*. a) Rates of *A. nidulans* infection of wildtype (Wt) *Arabidopsis* seeds from both the Columbia (Col) and Landsberg (Ler) ecotypes. Seeds were whole-plant-harvested and used about 850 days after flowering. N=3 for each treatment. b) Rates of *Aspergillus flavus* infection of *Arabidopsis* Wt Col and mutant *chs* seeds (in the Ler genetic background). Seeds were whole-plant-harvested and used about 371 days after flowering. N=5 for each treatment. c) Rates of *A. nidulans* infection of *Arabidopsis* Wt Col and mutant *chs* seeds. Seeds were whole-plant-harvested and used about 371 days after flowering. N=5 for each treatment. d) Rates of *A. nidulans* infection of *Arabidopsis* seeds mutant in the *erecta* (*er*) gene, in both the Col and Ler genetic backgrounds, compared to a *chs* positive control. Seeds were whole-plant-harvested and used about 70 days after flowering. N=5 for all treatments. All assays were carried out in the dark at 37°C for *A. nidulans*, or at 28°C for *A. flavus*. Quantification of infection is represented by the number of spores per seed, plus or minus the standard error of the mean (SEM). A two-tailed t-test with an alpha value of 0.05 was run, comparing each infection rate to that of the no-seed control (a) or to that of Wt Col (b, c, d). P-values below 0.05 (*) are indicated above each treatment. Each no-spore control was clear of infection (data not shown). Each experiment was repeated twice with similar results.
Figure 8 (previous page). *A. nidulans* infection of *Arabidopsis* seeds mutant in flavonoid biosynthetic pathway genes, compared to the infection rate of Wt Col seeds. All seeds were whole-plant-harvested. 

a) *chs* seeds, 371 days after flowering; N=5.  
b) *f3' h* seeds, 133 days after flowering; N=3.  
c) *dfir* seeds, 133 days after flowering; N=5.  
d) *fls* seeds, 260 days after flowering; N=3.  
e) *ldox* seeds, 260 days after flowering; N=3.  
f) *ban* seeds, 140 days after flowering; N=5.  
g) *tt12* seeds, 154 days after flowering; N=5.  
h) *aha10* seeds, 581 days after flowering; N=5.  
i) *tt15* seeds, 140 days after flowering; N=3.  
j) *gstf12* seeds, 140 days after flowering; N=3.  
k) *wip1* seeds, 847 days after flowering; N=3.  
l) *bhlh42* seeds, 140 days after flowering; N=4.  
m) *lac15* seeds, 581 days after flowering; N=5.  

A two-tailed t-test with an alpha value of 0.05 was run, comparing the infection rate of each mutant seed type to that of Wt Col seeds. P-values below 0.05 (*) are indicated above each treatment. All experiments were repeated a minimum of two times with similar results.
Figure 9. Rates of *A. nidulans* infection of laccase mutant seeds (*lac15* and *lac5*), compared to Wt Col and a *chs* positive control. Seeds were whole-plant-harvested and used 70 days after flowering. Each no-spore control was clear of infection (data not shown). N=3 for each treatment. A two-tailed t-test with an alpha value of 0.05 was run, comparing the infection rate of each mutant seed type to that of Wt Col seeds. P-values below 0.05 (*) are indicated above each treatment. The experiment was repeated twice with similar results.
Figure 10. Rates of *A. nidulans* infection of *chs* mutant seeds of different ages. Wt Col and younger *chs* seeds were used 50 days after flowering, and older *chs* seeds were used 350 days after flowering. All seeds were whole-plant-harvested. Each no-spore control was clear of infection (data not shown). N=5 for each treatment. A two-tailed t-test with an alpha value of 0.05 was run, comparing the infection rate of each *chs* mutant seed cohort to that of Wt Col seeds. P-values below 0.05 (*) are indicated above each treatment. The experiment was repeated twice with the same seed cohorts, with similar results.
Figure 11. Timecourse of *A. nidulans* infection of *Arabidopsis* Wt Col and *chs* seeds at different ages of maturity (between zero and 140 days after flowering). Wt Col and *chs* seeds were marked weekly and were harvested silique-by-silique one week after peduncle collapse. The positive control was older *chs* seeds (about 645 days after flowering) that were whole-plant harvested. Even the oldest mature seeds of both Wt Col and *chs* remained resistant to *A. nidulans*, while the positive control *chs* seeds were mildly susceptible. Each no-spore control was clear of infection (data not shown). N=3 for each treatment.
a) $chs$ (silique-harvested) $\quad$ $chs$ (whole-plant-harvested)

b) Spores/Seed (+/- SEM)

<table>
<thead>
<tr>
<th></th>
<th>Wt Col (silique-harvested)</th>
<th>$chs$ (silique-harvested)</th>
<th>Wt Col (whole-plant-harvested)</th>
<th>$chs$ (whole-plant-harvested)</th>
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<tr>
<td>Spores/Seed</td>
<td>0E+0</td>
<td>1E+4</td>
<td>2E+4</td>
<td>6E+4</td>
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</tbody>
</table>


c) Percent of seeds

<table>
<thead>
<tr>
<th></th>
<th>$chs$ (silique-harvested)</th>
<th>$chs$ (whole-plant-harvested)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ND</td>
<td>Fully germinated</td>
<td>Failed to germinate</td>
</tr>
</tbody>
</table>

Legend:
- **Green**: Fully germinated
- **Light Green**: Radical emergence only
- **Black**: Failed to germinate
Figure 12 (previous page). Comparison of \textit{chs} seeds of the same age, but with different plant-care methods. \textbf{a}) A comparison of silique-harvested and whole-plant-harvested \textit{chs} seeds (about 315 days after flowering). The yellow silique-harvested seeds were harvested one week after peduncle collapse; the grey-yellow whole-plant-harvested seeds were harvested after eight months of drying on the parent plant. Image was acquired with a magnified iPad camera, with a dissecting pin separating the two seed cohorts. \textbf{b}) \textit{A. nidulans} infection rates of the \textit{chs} seeds pictured above. Each no-spore control was clear of infection (data not shown). N=5 for each treatment. A two-tailed t-test with an alpha value of 0.05 was run, comparing the infection rate of \textit{chs} mutant seeds to that of Wt Col seeds from the same cohort. P-values below 0.05 (*) are indicated above each treatment. The experiment was repeated two times, with similar results. \textbf{c}) Germination rates of the \textit{chs} seeds shown above. N=3, with 30-40 seeds per plate. The germination test was repeated twice with similar results. Data that was not collected is indicated as not done (ND).
**a)**

**b)**

- *chs (middles of branches)*
- *chs (ends of branches)*
- *chs (late-forming branches)*

**c)**

![Graph showing Spores/Seed (±/ SEM) for Wt Col, chs (yellow), and chs (late-forming)]

**d)**

![Bar chart showing Percent of seeds for ND, chs (yellow), and chs (late-forming)]

- **Fully germinated**
- **Radical emergence only**
- **Failed to germinate**

**e)**

- *chs (yellow)*
  - 97% Germination
- *chs (late-forming)*
  - 15% Germination
Figure 13 (previous page). Comparison of chs seeds from the same plant, but formed at different developmental stages of the parent plant. **a**) A yellow chs seed (left) compared to a smaller, darker chs seed (right). Both seed phenotypes can occur on a single chs parent plant. Image acquired using a magnified iPad camera. **b**) Dissection of a chs parent plant. Seeds from the middles of the parent plants’ main branches (*top*); Seeds from the last five siliques at the ends of the parent plants’ main branches (*middle*); Seeds from late-forming siliques, which often form in tight clusters after the plant has been taken off of water (*bottom*). The late-forming seeds are an average of 44% smaller than seeds from other branches of the plant. Seed sizes were measured using ImageJ surface area analysis. Images were acquired with a SpotBasic camera and software, under the same lighting and magnification. The scale bar represents one millimeter. **c**) Rates of *A. nidulans* infection of sorted chs seeds (645 DAF), with yellow seeds separated from the late-forming seeds, which are smaller and darker. Each no-spore control was clear of infection (data not shown). N=3 for each treatment. A two-tailed t-test with an alpha value of 0.05 was run, comparing the infection rate of each chs seed type to that of Wt Col seeds. P-values below 0.05 (*) are indicated above each treatment. Experiment was repeated twice with similar results. **d**) Germination rates of the sorted chs seeds. N=3 for each seed type, with 35-50 seeds per plate. Germination test was repeated twice with similar results. Data that was not collected is indicated as not done (ND). **e**) The chs seedlings from yellow seeds (left) grew well, with a diameter up to 1.3 centimeters after two weeks of growth. The chs seedlings from the late-forming seeds did not grow past three millimeters in diameter. The scale bar represents one centimeter.
Figure 14. Comparison of chs seeds with identical plant-care methods, but with different genetic backgrounds. Seeds were whole-plant-harvested after five weeks of drying, and were used in the assays 70 days after flowering. a) Rates of A. nidulans infection of four different chs mutant seeds compared to Wt Col and Wt Ler. Each no-spore control was clear of infection (data not shown). N=5 for each treatment. A two-tailed t-test with an alpha value of 0.05 was run, comparing the infection rate of each seed type to that of Wt Col seeds. P-values below 0.05 (*) are indicated above each treatment. Experiment was repeated twice with similar results. b) Germination rates of each seed type. N=3, with 20-60 seeds per plate. Experiment was repeated twice with similar results.
Figure 15. Inhibition of *A. nidulans* growth in the presence of Wt Col aqueous seed extract, compared to a no-extract (NE) water control. *A. nidulans* growth was quantified by spores/cm². The experiment was run for forty-five hours at 37°C, in the dark. N=5 for each treatment. A two-tailed t-test with an alpha value of 0.05 was run, comparing *A. nidulans* growth in the presence of water compared to growth in the presence of Wt Col seed extract. A p-value below 0.05 (*) is indicated. The experiment was repeated three times with new seed extract, with similar results.
Figure 16 (previous page). Variability of chs seed infection, germination, and chemical inhibition of A. nidulans. Comparisons are made between whole-plant-harvested (WPH) chs seeds of different ages, silique-harvested (SH) chs seeds, and bulk-harvested (BH) seeds that served as a positive control. a) Rates of A. nidulans infection of chs seeds compared to Wt Col seeds. Each no-spore control was clear of infection (data not shown). N=5 for each treatment. A two-tailed t-test with an alpha value of 0.05 was run, comparing the infection rate of each chs mutant seed type to that of Wt Col seeds. P-values below 0.05 (*) are indicated above each treatment. Experiment was repeated twice for each seed type, with similar results. b) Germination rates of chs and Wt Col seeds. N=3 for each seed type, with 30-70 seeds per plate. Germination test was repeated twice for each seed type, with similar results. Data that was not collected is indicated as not done (ND). c) Inhibitory properties of aqueous extracts from Wt Col and chs seeds, compared to a no-extract (NE) water control. The experiment was run for forty-five hours at 37°C, in the dark. N=5 for each treatment. A two-tailed t-test with an alpha value of 0.05 was run, comparing A. nidulans growth in the presence of chs seed extracts compared to A. nidulans growth in the presence of Wt Col seed extract. A p-value below 0.05 (*) is indicated above each treatment. The experiment was repeated twice with the same seed extracts, showing similar results.