Biotechnological approaches for improved disease resistance in soybean and wheat

by

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B.S., Fort Hays State University, 2009
M.S., Wichita State University, 2014

AN ABSTRACT OF A DISSERTATION

submitted in partial fulfillment of the requirements for the degree

DOCTOR OF PHILOSOPHY

Department of Plant Pathology
College of Agriculture

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Manhattan, Kansas

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Abstract

Increasing food production is required for the ever-increasing population. Reducing crop losses due to plant pathogens is a viable method of increasing this production. An estimated 12.5% of crop productivity is lost each year to plant pathogens. Here we explore two methods that utilize biotechnology for increasing resistance of common bread wheat (*Triticum aestivum* L.) to fungal pathogens, and soybean (*Glycine max* (L.) Merr.) to soybean cyst nematode (SCN; *Heterodera glycines* Ichinohe). Fungi are responsible for the largest amount of yield loss among plant pathogens. We have introduced six antifungal peptides (zeamatin, drosomycin, juruin, ARACIN, Ace-AMP1, and a wasabi gamma-thionin) into wheat via particle bombardment as single transgene lines along with a glufosinate resistance marker (*BAR*). Coding sequences (CDSs) were constitutively expressed via the pAHC17 plasmid. Lines expressing these genes were challenged with the ascomycete fungal pathogens *Pyrenophora tritici-repentis*, and *Fusarium graminearum*; as well as the basidiomycete fungal pathogen *Tilletia laevis*. Despite high levels of expression compared to housekeeping genes, these transgenes did not show enhanced resistance to these fungal pathogens. Zeamatin, drosomycin, and juruin CDSs were also subcloned into the soybean-optimized constitutive expression vector pGmubi. The other focus of this work looks at reducing reproductive success of SCN in the SCN/soybean pathosystem by exploiting SCN sex pheromones. SCN males have been shown to be attracted to females by several organic compounds, namely vanillic acid (VA). Here we engineer a pathway to produce VA in soybean in an attempt to mask females from males, thus lowering their reproductive success. Because of the long regeneration time required to recover transgenic soybean from particle bombardment of calli, preliminary work was conducted to show proof-of-concept. To check for inhibition of VA on soybean at SCN working concentrations, pouch

bioassays were performed on 13-day old soybean seedlings. Root length of these seedlings was not inhibited by VA. Greenhouse experiments were conducted where susceptible soybean were challenged with SCN in the presence of different concentrations of exogenously applied VA. These assays did not show a reduction in cyst or egg numbers on soybean roots. A 3-dehydroshikimate dehydratase (3DSD) from *Podospora anserina* and a catechol o-methyltransferase (COMT) from *Nicotiana tubacum* were chosen for converting the shikimate pathway compound 3-dehydroshikimic acid into VA *in planta*. A *Petunia x hybrida* chloroplast targeting sequence fusion of these CDSs were subcloned into the pGmubi vector. These plasmids were introduced into soybean via particle bombardment either co-bombarded with the hygromycin resistance conferring plasmid pHyg, or tri-bombarded. Two greenhouse experiments were performed where T_1 soybean expressing 3DSD, COMT, and 3DSD+COMT were challenged with SCN. These plants did not show a reduction in numbers of cysts harvested from roots. More work needs to be done to dismiss this concept by doing larger greenhouse experiments with T_2 seed and plants expressing transgenes need to be characterized for production of VA. These observations indicate that VA may not act as a sex pheromone for SCN and needs to be investigated further.

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Approved by:

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Harold N. Trick

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Dedication

To my Family, close Friends, God, and Country.

Chapter 1 - General Introduction

Soybean: biology, taxonomy, importance

Soybean (*Glycine max* (L.) Merr.) is a major crop grown throughout the world. Soybeans are grown in tropical, subtropical, and temperate climates. It is the fourth largest crop in area planted following wheat, maize, and rice. Soybean was first domesticated in east Asia approximately 3,000 years ago and was introduced into the United States by Samuel Bowen in 1765 (Ho 1969, Hymowitz and Shurtleff 2005). Since then, the United States has become the number one producer of soybean, producing about 132 million tons in 2017 (FAO data). Other top producing countries include Brazil, Argentina, and China. Soybean is the third largest crop of Kansas and generates $1.6 billion in annual revenue (USDA-NASS information, 10 yr. mean).

Soybeans are part of the legume family, Fabaceae, which include many other important crops species such as peanuts, alfalfa, chickpeas, and peas. The current taxonomic classification of *G. max* is shown in Figure 1.1 (ITIS). Binomial synonyms for soybean include *Dolichios sofa* L., *Dolichos soja* L., *Glycine angustifolia* Miq., *Glycine gracilis* Skvortsov, *Glycine hispida* (Moench) Maxim., *Glycine hispida* var. *brunnea* Skvortsov, *Glycine hispida* var. *lutea* Skvortsov, *Glycine mas*, *Glycine soja* (L.) Merr., *Phalseolus max* L., *Phaseolus max* L., *Soja angustifolia* Miq., *Soja hispida* Moench, *Soja japonica* Savi, *Soja max* (L.) Piper, *Soja soja* H. Karst., and *Soja viridis* Savi (Global Biodiversity Information Facility).

Domain	Eukaryota
Kingdom	Plantae
Division	Tracheophyta
Class	Magnoliopsida
Order	Fabales
Family	Fabaceae
Genus	*Glycine*
Specific epithet	*max*

Figure 1.1 Taxonomical classification for soybean

Soybean grows anywhere from 30 to 150 cm tall. This dicotyledonous C_3 plant has a pair of simple opposite leaves at the first node followed typically by alternate trifoliate leaves, but occasionally compound leaves with four or more leaflets are present. Soybean is propagated commercially by seed. The flowers of soybean are self-pollinated, white to purple, and develop as a raceme. Fruits borne from flowers are legume pods. Pods are often pubescent containing one to five seeds. Beans within soybean pods exhibit a wide range in color including green, yellow, brown and black. In the US, soybean is typically planted late April through May and harvested late in October to early November. Soybean is an excellent source of protein and oil. They are one of the few plants that are a complete source of essential amino acids (AA). This quality makes them ideal for imitation meats and food additives for nutritional fortification. It also makes soybean meal a good choice for livestock feed. The absence of lactose makes soybean an attractive alternative to dairy milk. By-products of soybean processing include biodiesel, lubricants, cleaning agents, and adhesives. This versatility of uses for soybean helps account for its popularity as a cash crop.

G. max has a diploid genome with an unusually large chromosome number (2n = 40) compared to other closely related legumes, which is thought to be the result of two whole genome duplication events around 41.6 million years ago (mya) and 15 mya (Schlueter, Dixon et al. 2004). The first complete sequence of the soybean genome was completed in 2008 by the

United States Department of Energy Joint Genome institute. Around 46,400 protein coding genes are thought to be present in its 1.1 gigabase genome. About 17% and 42% of its genome is made up of transposable elements (TE) and retrotransposons respectively (Schmutz, Cannon et al. 2010). The large portion of the soybean (and wheat) genome made up of redundant TEs means that transgenes are likely to find a home in these areas. This can be advantageous in that a unique phenotype due to a transgene is more likely to be a result of the transgene and not a knock-out of a native gene. It can also be deleterious for a transgene in that these regions are typically transcriptionally repressed through epigenetics, which can lead to transcriptional repression of the transgene.

Wheat: biology, taxonomy, importance

Wheat is the number one crop in area planted worldwide, surpassing maize, the next most planted, by 21 million acres (FAO data: 2018). Wheat is also the most planted crop in Kansas and generates 1.4 billion dollars in annual revenue for the state (USDA-NASS data: Five-year mean 2014-2018). It was first cultivated about 10,000 years ago, with the modern form appearing at about 9,000 years ago (Shewry 2009). Genetic analysis indicates that this modern form arose in the southeastern part of Turkey (Heun, Schäfer-Pregl et al. 1997). It has since spread throughout the world and is grown in temperate environments. Wheat is an annual C3 crop that thrives in cool environments but can also be grown under warm, dry conditions. It is typically classified into broad categories based on its planting season (winter/spring), hardness (soft/hard), and color (red/white). Spring wheat is planted early in spring and is harvested late in the summer. In comparison, winter wheat is planted in autumn and becomes established before going dormant through the winter. This long cold period known as vernalization is necessary for winter wheat varieties to develop to maturity. Plants are then harvested in the summer. The

longer growing period of winter wheat allows for greater yield potential. Qualities such as hardness and color of wheat affect what it is typically used for in baking. Genetic diversity of wheat and closely related species helps account for almost 30,000 varieties grown throughout the world today (Posner and Hibbs 1997).

Wheat has a hexaploid genome that comes from *Triticum urartu* (einkorn; A genome), *Aegilops speltoides* (B genome), and *Triticum tauschii* (D genome). These genomes diverged from a common ancestor about 5 mya (Appels, Eversole et al. 2018). Numerous diploid and tetraploid progenitors of wheat are still grown throughout the world today due to being better adapted for certain growing conditions. The modern taxonomical classification for bread wheat is given in Figure 1.2 (ITIS). Other important grains that are considered wheat and are still grown include durum, kamut, einkorn, emmer, and spelt (Shewry 2009). *Triticum vulgare* L. is a common synonym for wheat that is commonly encountered in contemporary literature.

Domain	Eukaryota
Kingdom	Plantae
Phylum	Tracheophyta
Class	Magnoliopsida
Order	Poales
Family	Poaceae
Genus	*Triticum*
Specific epithet	*aestivum*

Figure 1.2 Taxonomical classification of modern wheat

The 21-chromosome genome of wheat has a size of about 14Gb, about 85% of which is made up of active or defunct TEs (Appels, Eversole et al. 2018). DNA transposons make up 16.5% of these elements and 67.5% come from retrotransposons. Of the total TE in wheat, Gypsy long terminal repeat retrotransposons make up the largest class of about 47%. Transposable elements from 505 families are found in the genome of wheat. Wheats genome is predicted to have anywhere from 105-269 thousand protein coding genes, with 62% of a subset

of genes from wheat have at least one homolog in each sub-genome (Appels, Eversole et al. 2018, UniProt 2020).

As for its anatomical features, wheat can grow anywhere from 30 to 152 cm in height; however, shorter varieties are popular due to reduced lodging. Like most monocots their leaves have parallel veins. The inflorescence of wheat is called a spike or less commonly a head or ear. The spike is made up of a central rachis that bears separate flowering groups known as spikelets. Each spikelet is made up of a rachilla that bears flowers known as florets. Wheat florets are comprised of an outer lemma, inner palea, a pistil with a single carpel, and three stamens. Two to four florets of each spikelet are typically fertile and develop into a fruit. The fruit of wheat is a caryopsis and is commonly referred to as a grain, kernel, or berry.

Grain of wheat is the primary part of the plant consumed by humans, making up a major source of calories consumed in the world, about 20% (Kumar, Yadava et al. 2011). Wheat also accounts for a substantial amount of dietary protein, fiber, fat, minerals (zinc and iron), and vitamins (thiamine and vitamin-B). The end product of wheat flour depends primarily on its protein content. This can vary anywhere from 7-22% depending on the variety of wheat, but generally ranges from 10-15% (Shewry and Hey 2015). High protein flour has good elastic properties that makes it suitable for making breads while low protein content flour is used for animal feed and other products. About 80% of protein found in wheat flour is comprised of prolamin storage proteins, which include glutin proteins that are responsible for dough plasticity and strength (Seilmeier, Belitz et al. 1991).

In addition to basic to providing essential dietary nutrients, dietary fiber from wheat has been shown to have a number of cardio-metabolic and colo-rectal health benefits including reduced chances of stroke, type 2 diabetes, colon and rectal cancers (Reviewed in: Shewry and

Hey 2015). Despite these health benefits, wheat has been associated with several diseases including celiac disease, dermatitis herpetiformis, baker's asthma, wheat-dependent exercise-induced anaphylaxis, atopic dermatitis, urticaria, anaphylaxis (Reviewed in: Shewry 2009).

Pathogens represent a major source of potential yield loss for both soybean and wheat. Current genetic resistance in these crops to some fungi and nematodes is sufficient; however, the biological nature of pathogens means they are continuously evolving to overcome this resistance. Additionally, there are no sources of genetic resistance to some fungal pathogens of soybean and wheat that are sufficient. Tradition methods for introducing resistance in plants, such as breeding, rely on a limited genetic pool and can be time consuming. Biotechnology is a viable method for expanding the genetic pool from which we can draw upon and can allow better control of resistance genes.

Biotechnology of soybean and wheat

To common methods are used to introduce genes from outside sources into plants. This includes particle bombardment and *Agrobacterium*-mediated transformation. The earliest reports of transforming soybean were in 1988 using both these methods by independent groups (Hinchee, Connor-Ward et al. 1988, McCabe, Swain et al. 1988). Genetically modified (GM) soybean became commercially available in the US for the first time in 1996. Currently there is no commercially available transgenic soybean for use in the US that confers resistance to fungi or soybean cyst nematode (SCN); however, GM soybean is one of the few crops that has seen widespread adoption. In 2017, 50% of GM crops planted worldwide were soybean. In the same year, 94% of soybean planted in the United States were genetically modified (ISAAA 2017). Traits for these GM soy include herbicide tolerance, insect and drought resistance, increased yield, and altered oil profiles. These traits are often stacked in one variety. Herbicide tolerant

varieties allowed for use in the US include glufosinate, glyphosate, 2,4-D, dicamba, isoxaflutole, and imidazolinone (USDA-APHIS 2019). Insect resistant soybean varieties derive their resistance from insecticidal crystal (Cry) proteins originally produced from *Bacillus thuringiensis* (Bt) subsp. *kurstaki*. When expressed in soybean, Cry proteins are effective against lepidopteran pests by interacting with cell membrane proteins of midgut cells and causing rupturing. These proteins have been shown to be effective at controlling some nematodes; however, there is no evidence that Cry proteins are effective against SCN and persistent SCN infestations in the presence of predominantly Bt soybean in fields would corroborate with this (Wei, Hale et al. 2003). As for altering oil profiles in soybean with the use of biotechnology, varieties have been developed with increased oleic acid content through the use of sense suppression and RNA interference (RNAi) as well as varieties with increased omega-3 fatty acids via transgene introduction (Pavely, Fedorova et al. 2007, George, Rogan et al. 2009, Jenkins, George et al. 2009). More recent GM traits to be deregulated for commercial use in the US include two varieties of soybean with increased yield. These varieties confer high yield through expression of recombinant transcription factor or recombinant proteins that interact with native soybean transcription factors (Bell, Best et al. 2011, Fazio, Redenbaugh et al. 2017). In a twenty year time span from 1996-2016, GM soybean accounted for an additional $59.8 billion in farm income worldwide (Brookes and Barfoot 2018). It is clear from the predominance of GM soybean planted that biotechnology in the field is here to stay.

Implementation of GM wheat in the agricultural setting has been less successful compared to soybean. As of yet, there are no commercialized varieties of GM wheat approved for use in the US (USDA-APHIS 2019). Successful transformation of wheat followed soon after the transformation of soybean when Vasil et al. (1992) recovered glufosinate resistant plants

from particle bombardment of wheat calli. Because of its evolved ability to efficiently transform dicot plants, *Agrobacterium*-mediated transformation of wheat did not happen until 1997 (Cheng, Fry et al.). This technology has been used to show proof of concept for improving wheat in a number of ways. For example, extensive work has been done to increase breadmaking qualities of wheat by addition of native gluten genes through the use of transgenics (Altpeter, Vasil et al. 1996, Lucrecia Alvarez, Gómez et al. 2001, Blechl, Lin et al. 2007). Also, nutritional quality of wheat has been improved by, among other things, increasing vitamin content and amino acid composition of seed (Tamas, Kisgyorgy et al. 2009, Wang, Zeng et al. 2014). One group has shown to improve growth qualities of wheat under adverse conditions such as heat stress through a variety of different mechanisms (Fu, Momcilovic et al. 2008, Tian, Talukder et al. 2018). Moreover, transgenic wheat has shown to be an effective method at reducing disease severity of several types of pathogen. For example, Rupp et al. (2019) exploited RNAi to increase resistance to several viral pathogens. As for fungal pathogens, numerous transgenic methods have been developed to reduce or control disease severity. Overexpression of recombinant chitinases have been used to reduce severity or control pathogens such as wheat rusts and *Fusarium graminearum* under field conditions (Shin, Mackintosh et al. 2008, Eissa, Hassanien et al. 2017). In another transgenic approach, priming of plant resistance machinery before fungal infection showed to be an effective method of pathogen control in wheat (Makandar, Essig et al. 2006). Despite these and other proven methods for improving wheat production and utilization, GM wheat remains absent in fields due to several socioeconomic reasons. Dire situations for new sources of resistance, such as a large-scale epidemic of wheat blast, could herald the use of GM wheat on the large scale.

Here we attempt to introduce novel sources of resistance to fungal pathogens in soybean and wheat by exploiting biotechnology. These new sources of resistance come in the form of antimicrobial peptides and are often effective against a broad range of species. We also attempt to disrupt the lifecycle of the number one pathogen of soybean, SCN, by producing a nematode sex pheromone in soybean via a synthetic metabolic pathway.

Works Cited

Altpeter, F., V. Vasil, V. Srivastava and I. K. Vasil (1996). "Integration and expression of the high-molecular-weight glutenin subunit 1ax1 gene into wheat." Nature Biotechnology **14**(9): 1155-1159.

Appels, R., K. Eversole, N. Stein, C. Feuillet, B. Keller, J. Rogers, C. J. Pozniak, F. Choulet, A. Distelfeld, J. Poland, G. Ronen, A. G. Sharpe, O. Barad, K. Baruch, G. Keeble-Gagnère, M. Mascher, G. Ben-Zvi, A.-A. Josselin, A. Himmelbach, F. Balfourier, J. Gutierrez-Gonzalez, M. Hayden, C. Koh, G. Muehlbauer, R. K. Pasam, E. Paux, P. Rigault, J. Tibbits, V. Tiwari, M. Spannagl, D. Lang, H. Gundlach, G. Haberer, K. F. X. Mayer, D. Ormanbekova, V. Prade, H. Šimková, T. Wicker, D. Swarbreck, H. Rimbert, M. Felder, N. Guilhot, G. Kaithakottil, J. Keilwagen, P. Leroy, T. Lux, S. Twardziok, L. Venturini, A. Juhász, M. Abrouk, I. Fischer, C. Uauy, P. Borrill, R. H. Ramirez-Gonzalez, D. Arnaud, S. Chalabi, B. Chalhoub, A. Cory, R. Datla, M. W. Davey, J. Jacobs, S. J. Robinson, B. Steuernagel, F. van Ex, B. B. H. Wulff, M. Benhamed, A. Bendahmane, L. Concia, D. Latrasse, J. Bartoš, A. Bellec, H. Berges, J. Doležel, Z. Frenkel, B. Gill, A. Korol, T. Letellier, O.-A. Olsen, K. Singh, M. Valárik, E. van der Vossen, S. Vautrin, S. Weining, T. Fahima, V. Glikson, D. Raats, J. Číhalíková, H. Toegelová, J. Vrána, P. Sourdille, B. Darrier, D. Barabaschi, L. Cattivelli, P. Hernandez, S. Galvez, H. Budak, J. D. G. Jones, K. Witek, G. Yu, I. Small, J. Melonek, R. Zhou, T. Belova, K. Kanyuka, R. King, K. Nilsen, S. Walkowiak, R. Cuthbert, R. Knox, K. Wiebe, D. Xiang, A. Rohde, T. Golds, J. Čížková, B. A. Akpinar, S. Biyiklioglu, L. Gao, A. N'Daiye, M. Kubaláková, J. Šafář, F. Alfama, A.-F. Adam-Blondon, R. Flores, C. Guerche, M. Loaec, H. Quesneville, J. Condie, J. Ens, R. Maclachlan, Y. Tan, A. Alberti, J.-M. Aury, V. Barbe, A. Couloux, C. Cruaud, K. Labadie, S. Mangenot, P. Wincker, G. Kaur, M. Luo, S. Sehgal, P. Chhuneja, O. P. Gupta, S. Jindal, P. Kaur, P. Malik, P. Sharma, B. Yadav, N. K. Singh, J. P. Khurana, C. Chaudhary, P. Khurana, V. Kumar, A. Mahato, S. Mathur, A. Sevanthi, N. Sharma, R. S. Tomar, K. Holušová, O. Plíhal, M. D. Clark, D. Heavens, G. Kettleborough, J. Wright, B. Balcárková, Y. Hu, E. Salina, N. Ravin, K. Skryabin, A. Beletsky, V. Kadnikov, A. Mardanov, M. Nesterov, A. Rakitin, E. Sergeeva, H. Handa, H. Kanamori, S. Katagiri, F. Kobayashi, S. Nasuda, T. Tanaka, J. Wu, F. Cattonaro, M. Jiumeng, K. Kugler, M. Pfeifer, S. Sandve, X. Xun, B. Zhan, J. Batley, P. E. Bayer, D. Edwards, S. Hayashi, Z. Tulpová, P. Visendi, L. Cui, X. Du, K. Feng, X. Nie, W. Tong

and L. Wang (2018). "Shifting the limits in wheat research and breeding using a fully annotated reference genome." Science **361**(6403): eaar7191.

Bell, E., T. C. Best, A. H. Culler, E. C. Dickinson, J. C. Goley, T. O. Koyejo, K. E. Niemeyer, J. R. Phillips, G. J. Rogan, A. Skipwith, H. Su, B. M. Baltazar, L. D. Barberis, P. L. Bommireddy, C. R. Brown, D. B. Carson, F. G. Dohleman, M. Elrod, A. Evans, A. C. Fitzgerald, C. W. Garnaat, T. Geng, S. W. Hoi, K. M. Huizinga, N. Ivleva, T. A. Kaempfe, M. S. Kock, K. R. Lawry, K. J. Mathis, S. A. Nolte, S. Paul, S. L. Phillips, C. Jiang, A. Silvanovich, D. Stojsin, S. J. Tauchman, Q. Tian, H. Tu, J. L. Vicini and R. Wang (2011). Petition for the determination of nonregulated status for mon 87712 soybean. B. R. S. USDA-APHIS, Monsanto. **11-202-01p**.

Blechl, A., J. Lin, S. Nguyen, R. Chan, O. Anderson and F. Dupont (2007). "Transgenic wheats with elevated levels of dx5 and/or dy10 high-molecular-weight glutenin subunits yield doughs with increased mixing strength and tolerance." Journal of Cereal Science **45**: 172-183.

Brookes, G. and P. Barfoot (2018). "Farm income and production impacts of using gm crop technology 1996–2016." GM Crops & Food **9**(2): 59-89.

Cheng, M., J. E. Fry, S. Pang, H. Zhou, C. M. Hironaka, D. R. Duncan, T. W. Conner and Y. Wan (1997). "Genetic transformation of wheat mediated by agrobacterium tumefaciens." Plant Physiology **115**(3): 971.

Eissa, H. F., S. E. Hassanien, A. M. Ramadan, M. M. El-Shamy, O. M. Saleh, A. M. Shokry, M. Abdelsattar, Y. B. Morsy, M. A. El-Maghraby, H. F. Alameldin, S. M. Hassan, G. H. Osman, H. T. Mahfouz, G. A. Gad El-Karim, M. A. Madkour and A. Bahieldin (2017). "Developing transgenic wheat to encounter rusts and powdery mildew by overexpressing barley chi26 gene for fungal resistance." Plant Methods **13**(1): 41.

Fazio, G., K. Redenbaugh, J. Alloatti, C. Blackford, M. Burachik, M. Chiozza, C. Dezar, I. Dicely, D. Facciotti, M. Gadisman, Z. X. Lu, E. Marchionni, P. Miranda, B. Preiss, M. Rivero, D. J. Schaaf, W. Skinner, F. Tarico and M. Vazquez (2017). Petition for determination of non-regulated status for the new plant variety hb4 soybean (ind-00410-5) intended for environmental release and food and feed use. B. R. S. USDA-APHIS, Verdeca. **17-223-01p**.

Fu, J., I. Momcilovic, T. E. Clemente, N. Nersesian, H. N. Trick and Z. Ristic (2008). "Heterologous expression of a plastid ef-tu reduces protein thermal aggregation and enhances co2 fixation in wheat (*triticum aestivum*) following heat stress." Plant Mol Biol **68**(3): 277-288.

George, C., G. J. Rogan, G. A. Bannon, J. D. Coyler, R. C. Dobert, D. A. Goldstein, B. G. Hammond, M. J. HOrak, D. L. Kendrick, J. M. Kronenberg, T. C. Lee, J. D. Masucci, B. Packer, E. A. Rice, J. F. Rice, R. W. Schneider, R. S. Sidhu and A. Silvanovich (2009). Petition for the determination of nonregulated status for mon 87769. B. R. S. USDA-APHIS, Monsanto. **09-183-01p**.

Global Biodiversity Information Facility. "Glycine max (l.) merr." Retrieved 1/14/20, from https://www.gbif.org/species/5359660.

Heun, M., R. Schäfer-Pregl, D. Klawan, R. Castagna, M. Accerbi, B. Borghi and F. Salamini (1997). "Site of einkorn wheat domestication identified by DNA fingerprinting." Science **278**(5341): 1312.

Hinchee, M. A. W., D. V. Connor-Ward, C. A. Newell, R. E. McDonnell, S. J. Sato, C. S. Gasser, D. A. Fischhoff, D. B. Re, R. T. Fraley and R. B. Horsch (1988). "Production of transgenic soybean plants using agrobacterium-mediated DNA transfer." Bio/Technology **6**(8): 915-922.

Ho, P.-T. (1969). "The loess and the origin of chinese agriculture." The American Historical Review **75**(1): 1-36.

Hymowitz, T. and W. R. Shurtleff (2005). "Debunking soybean myths and legends in the historical and popular literature." Crop Science **45**(2): 473-476.

ISAAA (2017). Global satus of commercialized biotech/gm crops in 2017: Biotech crop adoption surges as economic benefits accumulate in 22 years. Ithaca, NY, International Service for the Acquisition of Agri-biotech Applications.

ITIS. Retrieved March 16th, 2020, from http://www.itis.gov.

Jenkins, D. J., C. George, G. J. Rogan, R. S. Sidhu, M. A. Wideman, T. Armstrong, G. A. Bannon, B. Baltazar, L. Burzio, C. W. Carrera, J. Colyer, A. W. Crawford, R. C. Dobert, J. M. Dunn, T. Geng, M. Giovannini, K. C. Glenn, G. Hartnell, M. J. Horak, K. Huizinga, S. Kapadia, D. L. Kendrick, K. J. Klug, C. A. Lawrence, S. Lemke, D. Laundry, J. Masucci, J. A. McClure, B. Packer, A. Pan, T. Perez, J. Petrick, S. Phillips, W. P. Ridley, T. L. Reynolds, K. Robinson, M. Rowland, R. W. Schnieider, A. Silvanovich, A. Skipwith, Q. Tian, H. Tu, M. Wallendorf and C. Wang (2009). Petition for the determination of nonregulated status for improved fatty acid profile mon 87705 soybean. B. R. S. USDA-APHIS, Monsanto. **09-201-01p**.

Kumar, P., R. K. Yadava, B. Gollen, S. Kumar, R. K. Verma and S. Yadav (2011). "Nutritional contents and medicinal properties of wheat: A review." Life Sciences and Medicine Research **LMSR-22**.

Lucrecia Alvarez, M., M. Gómez, J. María Carrillo and R. H. Vallejos (2001). "Analysis of dough functionality of flours from transgenic wheat." Molecular Breeding **8**(1): 103-108.

Makandar, R., J. S. Essig, M. A. Schapaugh, H. N. Trick and J. Shah (2006). "Genetically engineered resistance to fusarium head blight in wheat by expression of *arabidopsis* npr1." Mol Plant Microbe Interact **19**(2): 123-129.

McCabe, D. E., W. F. Swain, B. J. Martinell and P. Christou (1988). "Stable transformation of soybean (*glycine max*) by particle acceleration." Bio/Technology **6**(8): 923-926.

Pavely, C., M. Fedorova and N. Weber (2007). Petition for the determination of nonregulated status for high oleic 305423 soybean. B. R. S. USDA-APHIS, Pioneer-DuPont. **06-354-01p**.

Posner, E. S. and A. N. Hibbs (1997). Wheat flour milling, American Association of Cereal Chemists, Inc. .

Rupp, J. S., L. Cruz, H. N. Trick and J. P. Fellers (2019). "Rnai-mediated silencing of endogenous wheat genes eif(iso)4e-2 and eif4g induce resistance to multiple rna viruses in transgenic wheat." Crop Science **59**(6): 2642-2651.

Schlueter, J. A., P. Dixon, C. Granger, D. Grant, L. Clark, J. J. Doyle and R. C. Shoemaker (2004). "Mining est databases to resolve evolutionary events in major crop species." Genome **47**(5): 868-876.

Schmutz, J., S. B. Cannon, J. Schlueter, J. Ma, T. Mitros, W. Nelson, D. L. Hyten, Q. Song, J. J. Thelen, J. Cheng, D. Xu, U. Hellsten, G. D. May, Y. Yu, T. Sakurai, T. Umezawa, M. K. Bhattacharyya, D. Sandhu, B. Valliyodan, E. Lindquist, M. Peto, D. Grant, S. Shu, D. Goodstein, K. Barry, M. Futrell-Griggs, B. Abernathy, J. Du, Z. Tian, L. Zhu, N. Gill, T. Joshi, M. Libault, A. Sethuraman, X.-C. Zhang, K. Shinozaki, H. T. Nguyen, R. A. Wing, P. Cregan, J. Specht, J. Grimwood, D. Rokhsar, G. Stacey, R. C. Shoemaker and S. A. Jackson (2010). "Genome sequence of the palaeopolyploid soybean." Nature **463**: 178.

Seilmeier, W., H. D. Belitz and H. Wieser (1991). "Separation and quantitative determination of high-molecular-weight subunits of glutenin from different wheat varieties and genetic variants of the variety sicco." Zeitschrift für Lebensmittel-Untersuchung und Forschung **192**(2): 124-129.

Shewry, P. R. (2009). "Wheat." Journal of Experimental Botany **60**(6): 1537-1553.

Shewry, P. R. and S. J. Hey (2015). "The contribution of wheat to human diet and health." Food and energy security **4**(3): 178-202.

Shin, S., C. A. Mackintosh, J. Lewis, S. J. Heinen, L. Radmer, R. Dill-Macky, G. D. Baldridge, R. J. Zeyen and G. J. Muehlbauer (2008). "Transgenic wheat expressing a barley class ii chitinase gene has enhanced resistance against *fusarium graminearum*." J Exp Bot **59**(9): 2371-2378.

Tamas, C., B. N. Kisgyorgy, M. Rakszegi, M. D. Wilkinson, M. S. Yang, L. Lang, L. Tamas and Z. Bedo (2009). "Transgenic approach to improve wheat (*triticum aestivum* l.) nutritional quality." Plant Cell Rep **28**(7): 1085-1094.

Tian, B., S. K. Talukder, J. Fu, A. K. Fritz and H. N. Trick (2018). "Expression of a rice soluble starch synthase gene in transgenic wheat improves the grain yield under heat stress conditions." In Vitro Cellular & Developmental Biology - Plant **54**(3): 216-227.

UniProt. (2020). "Proteomes - *triticum aestivum* (wheat)." Retrieved 3/28/20, 2020, from https://www.uniprot.org/proteomes/UP000019116.

USDA-APHIS. (2019). "Petitions for determination of nonregulated status." Retrieved 1/13/20, 2020, from https://www.aphis.usda.gov/aphis/ourfocus/biotechnology/permits-notifications-petitions/petitions/petition-status.

Vasil, V., A. M. Castillo, M. E. Fromm and I. K. Vasil (1992). "Herbicide resistant fertile transgenic wheat plants obtained by microprojectile bombardment of regenerable embryogenic callus." Bio/Technology **10**(6): 667-674.

Wang, C., J. Zeng, Y. Li, W. Hu, L. Chen, Y. Miao, P. Deng, C. Yuan, C. Ma, X. Chen, M. Zang, Q. Wang, K. Li, J. Chang, Y. Wang, G. Yang and G. He (2014). "Enrichment of provitamin a content in wheat (*triticum aestivum* l.) by introduction of the bacterial carotenoid biosynthetic genes crtb and crti." J Exp Bot **65**(9): 2545-2556.

Wei, J.-Z., K. Hale, L. Carta, E. Platzer, C. Wong, S.-C. Fang and R. V. Aroian (2003). "*Bacillus thuringiensis*; crystal proteins that target nematodes." Proceedings of the National Academy of Sciences **100**(5): 2760.

Chapter 2 - Plant Fungal Resistance via Recombinant Antifungal Peptides

Introduction

Fungi are the largest and most economically important group of pests to plants throughout the world. Historically, fungi have accounted for as much as 70% of yield losses due to pathogens (calculated from Adams, Bailey et al. 1965). Pathogenic fungi can also have widespread ecological effects. For example, the American chestnut tree (*Castanea dentata* (Marsh.) Borkh.) was once the dominant trees species of eastern United States hardwood forests. After the introduction of chestnut blight (*Cryphonectria paasitica* (Murr.) Barr) into the New York Zoological Garden in 1905, almost all wild American chestnut trees were destroyed within 50 years (Anagnostakis 1987). Extensive efforts are still being made to bring this tree back to forests around the world (Newhouse, Polin-McGuigan et al. 2014, Rigling and Prospero 2018). Plant pathogenic fungi can reach epidemic proportions due to their ability to produce large amounts of inoculum that can be carried for miles on the wind. During the 1970's the southern corn leaf blight (*Bipolar maydis* T.) epidemic in America reached its pinnacle, reduce corn yields in a single field by up to 100% (reviewed in Bruns 2017). This was largely due to extensive mono-culturing of a susceptible variety of corn. More recently, an epidemic of wheat blast (*Magnaporthe oryzae*) in Bangladesh reduced wheat yields by up to 100% (Islam, Croll et al. 2016). Not surprisingly, fungi have a major impact on local crops as well. The top two yield reducing diseases for wheat and soybean in Kansas are fungi (Jardine 2014, Appel, DeWolf et al. 2015). Fungi are responsible for reducing wheat yields in Kansas by approximately $80 million year (Calculated: USDA-NASS information; Appel, DeWolf et al. 2015).

Several methods can be used for controlling phytopathogenic fungi including the use of biocontrol, crop rotation, cultural practices, fungicide treatments, and natural genetic resistance. Because of variable efficacy for some of these practices, farmers typically rely on fungicide treatments and natural resistance for controlling plant pathogenic fungi in the field. In 2012 alone, $1.8 billion was spent on fungicides in the United States (Atwood and Paisley-Jones 2017). Some crops require multiple applications of fungicides within a single growing season for adequate protection. This can make fungicide use impractical when market values for crops become low. In addition, there are numerous cases of fungi developing resistance to fungicides (Fernández-Ortuño, Pérez-García et al. 2006, Banno, Fukumori et al. 2008, Yang, He et al. 2019). Given the importance of plant pathogenic fungi, novel sources of durable resistance need to be continually developed. In this research we introduce novel sources of proven resistance to fungi into wheat and challenge these lines against several historically and economically important phytopathogenic fungi. The fungi tested here include *Tilletia laevis*, causal agent of common bunt, *Fusarium graminearum*, causal agent of Fusarium head blight, and *Pyrenophora tritici-repentis*, causal agent of tan spot of wheat.

Smuts and bunts of plants have a great historical significance in agronomy and plant pathology. The earliest work done by Mathieu Tillet (1755), and later by Bénédict Prévost, was the first case to show a transmittable disease by a microorganism some 50 years before Koch came up with his postulates for the transmission of disease in animals. Smut and bunt fungi are a large group of basidiomycetes that infect around 4,000 plant species. The term smut comes from the dark sooty color of teliospore infested plant tissue while bunt may have come from the mispronunciation of burnt in reference to the same tissue (Duran and Fisher 1961). Common bunt (CB), the disease evaluated in the present study, was the most important plant pathogen of

wheat in the first half of the 20th century. In 1936 CB was responsible for reducing Kansas wheat yields by 10% (Haskell, Leukel et al. 1931). Contemporary times have not fared better for the disease. In the 1990's, 80-90% of Canadian wheat was lost to CB due to popular varieties having highly susceptible backgrounds from breeding (Gaudet and Laroche 1998).

The causal agents for CB are the species *Tilletia laevis* J.G. Kűhn (*T. laevis*) and *Tilletia tritici* (Bjerk.) G. Winter. The most obvious difference between the two is that *T. laevis* has smooth teliospore walls while *Tilletia tritici* has reticulated walls. Genetic evidence and their ability to hybridize has led some to question if the two species are distinct or actually variants of the same species (Russell and Mills 1994, Shi, Loomis et al. 1996). CB is found in all major wheat growing parts of the world. Alternative hosts for *T. laevis* include numerous wild grass species (Wilcoxson and Saari 1996).

Today, chemical protection is the primary method of controlling common bunt, so much so that breeding to introduce genetic resistance in elite lines has been neglected (Hoffmann and Waldher 1981). Prior to 1954, when the effective seed treatment of hexachlorobenzene was discovered, genetic resistance for common bunt was well studied. The major source of natural plant resistance relies heavily on the *Bt-10* gene. Besides this, numerous major and minor resistance genes are scattered throughout the genome of different wheat varieties (Fofana, Humphreys et al. 2008, Goates 2011). A study conducted by Lu et al. (2005) has implicated a ns-LTPs, a lipase, several pathogenesis-related proteins, and two R-genes as being responsible for resistance to CB. The popularity of organic farming and its lack of chemical pesticides has brought about a re-emergence of CB. Because of this, interest in plant resistance to CB has been rekindled in recent times.

The current taxonomical classification of T. laevis is given in Figure 2.1. Common synonyms for *T. laevis* include *Tilletia foetens* (Berk. & M. A. Curtis) J. Schrőt. in Cohn, *Tilletia foetida* (Wallr.) Liro, *Erysibe foetida* Wallr., Uredo caries DC, *Ustilago foetens* Berk. & M. A. Curtis. Other names commonly used for the disease include stinking bunt, stinking smut, covered smut, hill bunt, complete bunt, low bunt, and high bunt (Robert, Stegehuis et al. 2005).

Domain	Eukaryota
Kingdom	Fungi
Phylum	Basidiomycota
Class	Exobasidiomycetes
Order	Tilletiales
Family	Tilletiaceae
Genus	*Tilletia*
Specific epithet	*laevis*

Figure 2.1 Taxonomic classification of *Tilletia laevis*

T. laevis is a soilborne pathogen having a monocyclic disease cycle with only three spore types. Teliospores from the previous cycle of infection are deposited in the soil or on the surface of seed. These spores can remain viable in soil for up to ten years. As a testament to their hardiness, CB teliospores are capable of infection after passing through the digestive tract of several different ruminant animals (Johnsson 1990). *T. laevis* teliospores are smooth; globose, ovoid, or occasionally elongate, and 14-22 μm in diameter (Wilcoxson and Saari 1996). Teliospores germinate under wet, cold conditions to produce a promycelium. The promycelium differentiates at the tip to produce 4-16 primary sporidia (basidiospores). Two sporidia of opposite mating types then fuse to produce vegetative hyphae, infection hyphae, or secondary sporidia. Infection hyphae penetrate the coleoptile of seedlings and progress to the apical meristem intercellularly infecting the plant systemically. The hyphae must become established at the apical meristem before internode elongation or disease will not develop (Wilcoxson and Saari 1996). If infection is similar to the closely related *T. tritici*, hyphae can be found intra- and

intercellularly near the meristem area (Swinburne 1963). Little is known about the development of mycelium of *T. laevis* after establishment. Fischer and Holton (1957) commented "…it is very nearly impossible to develop an intelligible account of this phase of the biology of the smut fungi". Once the plants ovaries begin to development the fungus sporulates producing a kernel full of black, sooty teliospores. CB produces a distinctive fishy smell which is the result of the volatile compound trimethylamine. This smell gives the disease its alternate name "stinking smut". The teliospore filled kernels, called sori, fall to the ground or spores are released at harvest to complete the infection cycle.

Another fungal disease of wheat evaluated here is Fusarium head blight (FHB). Fusarium is a large genus of ascomycete fungi, many of which cause disease in animals and plants. FHB was first described in 1884 by W. G. Smith as "wheat scab". Later, in 1920, Dimitr Atanasoff used the term "Fusarium blight". Both terms are still used to this day. In the field, FHB is caused by a species complex of at least 16 fungi from the Fusarium genus (van der Lee, Zhang et al. 2015). In North America, central Europe, and Australia, *Fusarium graminearum* Schwabe (*F. graminearum*) [teleomorph: *Gibberella zeae* Schw. (Petch)] is considered the most important species causing FHB. Other Fusarium species commonly associated with FHB include *F. poae*, *F. sporotrichioides*, and *F. colmorum* in North America and *F. avenaceum* and *F. culmorum* in Europe (Stack 2003). *F. graminearum* is found in all major wheat growing areas. Historically it has caused large epidemics in the United States. In 1919 an outbreak caused an estimated loss of over 80 million bushels (Dickson, Johann et al. 1921). Modern times have not fared better for wheat growers regarding FHB. A 1993 epidemic in the United States caused an estimated loss of 156 million bushels of wheat (McMullen, Jones et al. 1997). Due to favorable conditions, FHB afflictions in Kansas are mainly restricted to central and eastern parts of the state where there is

more moisture and rotations to other susceptible crops such as corn are more common. In a particularly bad year for Kansas, 2015, losses in wheat yield due to FHB reached 14 million bushels, a loss representing $74 million (USDA , Appel, DeWolf et al. 2015). Lost revenue from FHB not only results from yield reductions, but also from rejected seed due to toxins produced by the *F. graminearum*. The toxin associated most with *F. graminearum* infected grain is vomitoxin, also known as deoxynivalenol (DON). As its name implies, DON induces vomiting in humans and animals when consumed at high concentrations. Other symptoms include diarrhea, abdominal pain, headaches, dizziness, and fever.

F. graminearum can be classified into two groups depending on the part of the plant it infects and the ability to form sexual fruiting bodies (i.e. perithecia). Group I strains are usually associated with crown diseases and rarely form perithecium while group II strains cause disease in aerial plant parts and readily form perithecium (Burgess, Wearing et al. 1975). *F. graminearum* can survive on crop residues residing on the soil surface for several years. Both conidia and ascospores are responsible for disease seen in field conditions (Markell and Francl 2003). Other crop species that are considered hosts of *F. graminearum* and contribute to epidemics include barley, oat, rye, triticale, corn, canary seed, flax, bean, lentil, and chickpea (Chongo, Gossen et al. 2001). It has been shown that *F. graminearum* can infect soybean and survive in soybean residues in fields, but no evidence has been shown that infested soybean material contributes to FHB of wheat (Anderson, Olechowski et al. 1988, Fernandez and Fernandas 1990). Saved seed from infected plants often results in reduced seedling emergence, seedling blight, and premature death of plants.

Common synonyms for *F. graminearum* that can be found in literature include *Gibberella zeae* (Schwein.) Petch, *Gibberella roseum* (Link) W.C. Snyder & E.M. Hans.,

Gibberella roseum (Link) W.C. Snyder & E.M. Hans. f. sp. *cerealis* (Cooke) W.C. Snyder & H.N. Hans, and *Gibbera saubinetii* (Mont.), *Gibberella saubinetii* (D. and M.), *Botryosphaeria sabinetii* (Mont.), *Gibbera pulicaris* (Fr.) f. *zeae maydis*, *Fusarium roseum* Autorum, *Fusarium tropicalis* Rehm, *Gibberella tritici* P. Henn. Figure 2.2 shows the current taxonomic classification of *Fusarium graminearum* (Robert, Stegehuis et al. 2005).

Domain	Eukaryota
Kingdom	Fungi
Phylum	Ascomycota
Class	Sordaromycetes
Order	Hypocreales
Family	Nectriaceae
Genus	*Fusarium*
Specific epithet	*graminearum*

Figure 2.2 Taxonomic classification of *Fusarium graminearum*

FHB is generally considered a monocyclic disease even though the pathogen can produce asexual macroconidia late in the year. Wind disperses ascospores of *F. graminearum* onto susceptible parts of plants and splashing rain disperses conidia. Ascospores are 3-septate, hyaline, and 3-5 x 17-25.5 µm in size and macroconidia are 3-7 septate, 2.5-5 x 35-62 µm in size, and bear a distinctive foot cell (Sutton 1982). Infection from either type of spore produces similar disease symptoms under experimental conditions (Stack 1989). FHB infection of wheat heads is favored by warm, humid conditions. Optimal growing temperatures for the fungus is 20-30°C. Although a moist period after initial inoculation is not required, moist periods longer than 24 hours favor infection (Sutton 1982). Wheat heads are most susceptible to *F. graminearum* at anthesis and remain susceptible until the soft dough stage. Anthers contain chemical stimulants for hyphal growth that include choline and betaine (Strange and Smith 1978). Hyphae develop intracellularly, in the apoplastic space, and on the surface of florets (Bushnell, Hazen et al.

2003). The fungus spreads through the wheat head along the rachis to other spikelets both apically and basely via vascular tissue (Tu 1930).

A number of methods are currently used to help control of FHB. These include cultural practices, application of fungicides, biocontrol agents, and plant resistance. Several triazole class compounds have been shown to work against *F. graminearum* including metconazole and prothioconazole (Wise 2016). However, the narrow window of flowering in wheat for fungicide efficacy, increased production costs, and safety to human health and the environment reduce the practicality of fungicide use. Natural host resistance is considered the safest and most effective form of controlling FHB; although, no variety of wheat has been shown to be immune to FHB. Over 350 varieties of spring wheat and 180 varieties of winter wheat showed varying levels of susceptibility to the pathogen (Scott 1927, Christensen, Stakman et al. 1929). Resistance that has been identified to date comes from more than 40 quantitative trait loci (QTL), with the highest level of resistance coming from the QTL *Fhb1* (Liu, Hall et al. 2009, Bai, Su et al. 2018). Long breeding times required to reduce linkage drag when transferring this QTL to elite varieties reduces the practicality of its widespread use. Several transgenic approaches have been shown to reduce severity of FHB including host-derived gene silencing and modification of plant defense pathways (Cheng, Song et al. 2015, Yu, Zhang et al. 2017). Overexpression of recombinant AMP's not tested here have shown to be a method for increased resistance to FHB in greenhouse and field experiments as well (Li, Zhou et al. 2011, Sasaki, Kuwabara et al. 2016).

Another fungal disease evaluated in this study was tan spot (TS) of wheat. TS is a disease caused by the necrotrophic ascomycete *Pyrenophora tritici-repentis* (Died.) Drechsler [Abbrev: Ptr; Anamorph: *Drechslera tritici-repentis* (Died.) Shoemaker]. The current taxonomical classification for Ptr is shown in Figure 2.3 (Robert, Stegehuis et al. 2005). The disease is also

known as yellow leaf spot. Ptr is also capable of infecting seed, causing discoloration resulting in diseases known as red/pink/dark smudges (Fernandez, DePauw et al. 2001). Ptr is found in all major wheat growing areas of the world. It is commonly cited as being able to reduce yields in wheat by up to 50% or more; however, this author has not found evidence for this resulting from natural epidemics. Field experiments with artificial inoculations performed by Shabeer & Bockus (1988) showed reductions in wheat yield by more than 50%. Documented cases of natural epidemics show yield reductions under favorable conditions in the field to be closer to 10-20% (Annone 1997, 2010). In Kansas, TS is the third most important fungal disease affecting wheat yield, ranking second in some years (Appel, DeWolf et al. 2015).

Domain	Eukaryota
Kingdom	Fungi
Phylum	Ascomycota
Class	Dothideomycetes
Order	Pleosporales
Family	Pleosporaceae
Genus	*Pyrenophora*
Specific epithet	*tritici-repentis*

Figure 2.3 Taxonomic classification of *Pyrenophora tritici-repentis*

Synonyms for Ptr include *Pleospora tritici-repentis* Died., *Pleospora trichostoma* (Fr.) Wint. f. sp *tritici-repentis* Died., and *Pyrenophora tritici-vulgaris* Dickson. Anamorph scientific names commonly encountered for Ptr include *Drechslera tritici-repentis* (Died.) Shoemaker, *Helminthosporium tritici-repentis* Died., *Drechslera tritici-vulgaris* (Nisikado) Ito, and *Helminthosporium tritici-vulgaris* Nisikado.

The primary inoculum for TS is overwintering ascospores surviving saprophytically on host residues. Infection from ascospores happens at flag leaf emergence in the fall for winter wheat, and throughout the growing season. Extended wet periods after flag leaf emergence is conducive for infection. Once infected, the leaves of susceptible hosts produce a chlorotic

diamond-shape with a brown spot at the center, with chlorotic lesions worsen with time. Reduced photosynthetic capacity from this chlorosis and necroses, as well as reduced tillering caused by Ptr infection, are responsible for yield reductions (Rees and Platz 1983). Conidiophores develop at these lesions producing conidia that start secondary infections throughout the growing season. Numerous grass species are alternate hosts for Ptr, particularly *Elymus repens*, and can contribute to epidemics (Reviewed: De Wolf, Effertz et al. 1998). Disease caused by Ptr is dependent on two proteinaceous effectors termed ToxA and ToxB. ToxA is responsible for necrosis while ToxB is responsible chlorotic symptoms in susceptible cultivars (Ballance, Lamari et al. 1989, Strelkov, Lamari et al. 1999). More recently, a third effector has been identified as ToxC (Effertz, Meinhardt et al. 2002). These toxins are responsible for five races of this pathogen, which can be distinguished on a differential set of wheat (Lamari, Sayoud et al. 1995).

Several methods are available to help control TS severity and subsequent yield losses. Crop rotation to a non-susceptible host for one year is capable of significant reducing disease severity (Bockus and Claassen 1992). Because infested wheat residue that resides on the soil surface is responsible for initial infection, plowing is another method at effectively controlling TS. The popularity of no-till systems for reducing soil erosion limits the practicality of this method of control though. In no-till systems, preventative and curative fungicides are available for controlling TS; however, their use is not always economically feasible.

Antimicrobial peptides

It has previously been shown that recombinant antimicrobial peptides (AMP) are a possible source of increased resistance to fungal plant pathogens when expressed in a variety of

plant systems (Reviewed in: Sinha and Shukla 2019). This research attempts to exploit this phenomenon by introducing AMP genes into wheat and soybean from non-native sources. AMP's are typically short AA sequences that have inhibitory effects on a variety of microbes. These peptides are typically 5-300 residues long, but can reach upwards of 1,200 AAs (Liu, Zhang et al. 2009, Waghu, Barai et al. 2016). AMP's were at first thought to only be cationic or amphipathic; however, more recent work has uncovered AMP's that are anionic as well (Lai, Liu et al. 2002). No single structure or motif defines AMP's. They can be made up of α-helices, β-pleated sheets, or a combination of the two. Many AMP's have multiple disulfide bonds for added stabilization. Ace-AMP1 for example, an AMP evaluated here, has 100% activity after being heated to 100°C for ten minutes (Cammue, Thevissen et al. 1995). AMP's can also take on more complex forms by conjugating with sugars, lipids, and nucleosides (Reviewed in: De Lucca and Walsh 1999).

AMP's have many modes of action. Early work for determining how AMP's inhibit microbial growth focused mainly on their ability to perturb cell walls and membranes. These polypeptides accomplish this in a number of different ways (Reviewed in: Haney, Mansour et al. 2017). Along with disturbing cell walls and membranes, AMPs are also capable of inhibiting the formation of cell walls (Pérez, Rosa et al. 1981, McCarthy, Troke et al. 1985). A less common mode of action for AMPs is their attachment to cell wall surfaces creating nanonets to entangle bacteria (Chu, Pazgier et al. 2012). More recent research has uncovered AMP's that act intracellularly in the target organism, affecting DNA, RNA, and protein synthesis (Nicolas 2009, Mardirossian, Grzela et al. 2014). Furthermore, there is at least one example of a single AMP having different mechanisms-of-action on different species (El-Mounadi, Islam et al. 2016). The diversity of these small peptides comes from the sheer number identified. Numerous databases

for AMP's exist (Wang, Li et al. 2008, Pirtskhalava, Gabrielian et al. 2016). One of the largest of which contains sequence, structure, and target species information for more than 8,000 (Waghu, Barai et al. 2016).

AMPs are produced by almost every type of living organism including insects, reptiles, plants, and mammals. Interestingly, even microorganisms like bacteria and fungi produce these compounds. Bacterial AMPs, known as bacteriocins, not only have inhibitory against fungi, but other closely related bacteria. For example, certain strains of *Escherichia coli* (*E. coli*) produce bacteriocins that act against susceptible individuals of the same species to gain a competitive advantage (Gratia 1925). Likewise, fungi produce AMP's that inhibit the growth of other fungi. For example, an AMP from the fungal plant pathogen *Fusarium graminearum* has been shown to have inhibitory effects on other species of *Fusarium* (Patiño, Vázquez et al. 2018). Other AMPs have a much broader target range. Limenin, a defensin from shelf beans, is able to inhibit growth of several different bacteria as well as fungi (Wong and Ng 2006).

Like AMP's found in other types of organisms, AMP's produced by plants are often cysteine-rich and are grouped into families consisting of thionins, defensins, hevein-like peptides, knottins, lipid transfer proteins, α-hairpinins, snakins, vicillin-like peptides, and others not fitting into these families. The terms thionins and defensins are often used interchangeably, with thionin being more commonly used for plants. Non-cysteine rich AMP's are produced by plants as well and include glycine-rich, glycine and histidine-rich peptides, and peptides less than 10 AAs (Goyal and Mattoo 2014, Tam, Wang et al. 2015). Plant AMP's can be found in many types of tissue, including seeds, stems, leaves, and roots (Sharma and Lonneborg 1996, Park, Park et al. 2000, de Beer and Vivier 2011). The number of AMPs produced by a single plant can

constitute a significant amount of its genome. The small model plant Arabidopsis for example is predicted to produce over 300 different AMPs (Silverstein, Graham et al. 2005).

Native wheat and soybean AMPs

AMPs from wheat were first identified as early as 1976. The road to their discovery started as early as 1895 when brewers recognized adding wheat flour to fermentations would inhibit brewers' yeast (Hernandez-Lucas, Fernandez de Caleya et al. 1974). There are currently about 43 known or predicted AMPs produced in wheat ranging from 23-225 AAs (compiled from APD3, CAMPr3, DRAMP, and DBAASP databases). Thaumatin-like (syn. PR5) and purothionin type peptides make up the majority of these AMPs. Purothionins of wheat are particularly well characterized. Like other AMPs, purothionins are cysteine-rich and have inhibitory activity against bacteria and yeast (Fernandez de Caleya, Gonzalez-Pascual et al. 1972, Hernandez-Lucas, Fernandez de Caleya et al. 1974). Because they are particularly well characterized, wheat proteins are an attractive source for increased pathogen resistance in other plant systems. Krishnamurthy et al. (2001) have shown overexpression of a wheat puroindoline in rice increases resistance to several phytopathogenic fungi. Thaumatin-like peptides are a large family of proteins found throughout the plant kingdom and other types of organisms (Reviewed in: Liu, Sturrock et al. 2010). They get their name from a sweet tasting protein produced by a plant found in Africa, *Thaumatococcus daniellii*. In wheat, expression of thaumatin-like proteins have been shown to be induced by fungal infection (Wang, Tang et al. 2010).

Knowledge of soybean AMPs is still in its infancy compared to wheat. Only a few studies have been conducted with soybean AMPs and they began to appear two decades or later after the first wheat AMP studies (Graham, Burkhart et al. 1992, Vasconcelos, Morais et al. 2008). There are currently about 51 known or predicted AMPs from soybean ranging in size from 25-346 AA

(compiled from APD3, CAMPr3, DRAMP, and DBAASP databases). A large majority of these 51 proteins, 43, are predicted thumatin-like proteins. The native proteins found in both soybean and wheat were taken into account for choosing recombinant genes to add to both of these plant systems.

Recombinant antimicrobial peptides evaluated

Besides evaluating novelty of transgene sequences to be added to soybean and wheat, several other factors were considered. During initial characterization of new AMPs they are often tested against model fungi to determine a dose dependence response for inhibition. For proof-of-concept, it would be desirable for a recombinant AMP to have a low effective concentration. Also, since the purpose of the final product of these genetically modified (GM) plants is human consumption, it would be advantageous that the recombinant AMPs do not pose a risk to human health. A number of methods have been used to evaluate this including mouse studies, checking for erythrocytes lysis, and sequence analysis compared to known toxins and allergens (Jang, Lee et al. 2007, Dimitrov, Flower et al. 2013). Based on these factors, the AMPs discussed below were chosen for introduction to wheat and other plant systems.

Juruin

Juruin is an AMP that is naturally found in the venom of the Amazonian Pink Toe spider (*Avicularia juruensis*). This tarantula is commonly sold in pet stores and its venom is considered non-toxic to humans. Juruin was shown to be non-hemolytic against human erythrocytes at antifungal working concentrations (Ayroza, Ferreira et al. 2012). As for its antifungal properties, juruin was shown to have inhibitory effects on *Candida albicans* at concentrations as low as 2.5-5μM and *Aspergilus niger* at 10μM (Ayroza, Ferreira et al. 2012). It has a predicted molecular

weight of 4.00 kDa. Juruin is a cysteine rich, 38 AA peptide with three disulfide bridges. The array of disulfide bridges is identical to all cystine knot containing peptides from spider venom (Escoubas and Rash 2004). Very little research has been done with this AMP. Its nucleic acid sequence and three-dimensional structure have yet to be elucidated. The exact mechanism of action for this peptide has yet to be determined, but its high sequence similarity to other spider toxins suggests that may inhibit voltage-gated ions channels (Ayroza, Ferreira et al. 2012). To date there are no cases of recombinant production of juruin.

Drosomycin

Drosomycin is a well characterized AMP that is naturally produced by the common fruit fly (*Drosophlia melanogaster*). First identified in 1994, drosomycin is translated as a 70 AA preprotein and processed into a truncated 44 AA active form (Fehlbaum, Bulet et al. 1994). The mature peptide has a predicted molecular weight (MW) of 4,897 Da. This AMP is part of a multigene family made of up seven different members. Although these genes are derived from gene duplication and have high sequence similarity, each has its own spectrum of antifungal activity with drosomycin having the broadest range (Yang, Wen et al. 2006). The exact mechanism of action is not known for drosomycin. Its secondary structure includes three β-pleated sheets and one α-helix. It resembles plant defensins with a cysteine-stabilized α-helix/β-pleated sheet motif (Landon, Sodano et al. 1997). The disulfide bridge arrangement for this AMP was determined from recombinant drosomycin produced from *Saccharomyces cerevisiae* (Michaut, Fehlbaum et al. 1996). Several labs have produced functional heterologous drosomycin from *E. coli* (Yuan, Gao et al. 2007, Zhang and Zhu 2010). Fehlbaum et al. have shown drosomycin inhibits spore germination of *Neurospora crassa* by 50% at 0.6μM and the phytopathogen *Fusarium oxysporum* at 4.2 μM (Fehlbaum, Bulet et al. 1994). These authors also

reported this AMP to have no hemolytic activity for bovine erythrocytes, which suggests it is safe for consumption by animals and humans. Common gene synonyms and abbreviations for drosomycin include *BcDNA:LP03851*; *CG10810*; *Crp*; *DIM 19*; *DIM 21*; *Dmel\CG10810*; *dmy1; drm*; *Drm*; *DRO*; *drom*; *Drom*; *DROM*; *dros*; *Dros*; *DROS*; *Droso*; *drs*; *DRS*; *drsm*; *IM21*.

Wasabi Gamma-Thionin

The wasabi (*Wasabia japonica* (Miq.) Matsum) AMP used in this research is classified as a gamma-thionin due to its high sequence similarity to other plant thionin-type defensins. Like other thionins, it is cysteine-rich. It is produced as an 80 AA preprotein with a 29 AA export signal sequence. The predicted MW for the mature peptide is 5.72 kDa. The three-dimensional structure of wasabi gamma-thionin has yet to be elucidated. Purified wasabi gamma-thionin from transient expression in *Nicotiana benthamiana* was shown to inhibit the phytopathogenic fungi *Magnaporthe grisea* at a concentration as low as 5µg mL^{-1} and *Botrytis cinerea* at 20µg mL^{-1} (Saitoh, Kiba et al. 2001). Transgenic rice (*Oryza sativa*) expressing this AMP showed increased resistance to *Magnaporthe grisea in vivo* (Kanzaki, Nirasawa et al. 2002). There have been no reported cases of expression of this AMP in microbes. Saitoh et al. (2001) reported attempting to express this AMP in *E. coli*, but were unsuccessful presumable due to toxicity to the bacteria.

Ace-AMP1

The AMP Ace-AMP1 (Ace) is one of the more well studied AMP's used in this research. Ace is naturally found in high quantity in onion (*Allium cepa*) seeds. It is translated as a 132 AA preprotein, with a 27 AA N-terminal apoplastic signal sequence and a 12 AA C-terminal sequence that is predicted for targeting to the vacuole (Tassin, Broekaert et al. 1998). The 93 AA mature peptide of Ace has four α-helices stabilized by four disulfide bridges. Its three-dimensional structure has been predicted by Tassin et al. (1998) through ^{1}H-NMR and molecular

modeling. The exact subcellular location of Ace remains to be determined; however, Narhari Patkar et al. (2006) showed functional, recombinant Ace lacking the C-terminus localized to the apoplast. Numerous studies with transgenic plants were the C-terminus is retained show good inhibition against filamentous fungal phytopathogens. Cammue et al. (1995) showed Ace to have a strong inhibitory effect against several plant pathogenic fungi compared to other plant AMP's with high sequence similarity. This includes a pathogen tested here, *Pyrenophora tritici-repentis*, but under *in vitro* conditions. Concentrations they found for inhibiting fungal growth by 50% (IC_{50}) were as low as 250 ng mL^{-1}, but often doubled in media with greater ionic strength (Cammue, Thevissen et al. 1995). Recombinant Ace produced from *E. coli* has been shown to be affective against the plant pathogenic fungi *Alternaria solani*, *Fusarium oxysporum* f. sp. *vasinfectum*, and *Verticillium dahliae* (Wu, He et al. 2011). Transgenic geranium, rose, banana, and rice expressing Ace have been developed and were shown to have increased resistance to fungal plant pathogens as well (Bi, Cammue et al. 1999, Li, Gasic et al. 2003, Patkar and Chattoo 2006, Sunisha, Sowmya et al. 2019).

Aracin1

Aracin1 is an AMP that is naturally expressed at high levels in tissues infected by fungi in *Arabidopsis thaliana* (Neukermans, Inze et al. 2015, Klepikova, Kasianov et al. 2016). It is found in tandem with another AMP with high sequence similarity. Like most of the other AMPs used in this study, Aracin1 has a predicted export signal sequence at its N-terminus. Neukermans et al. (2015) showed a GFP fusion of Aracin1 localized to the endoplasmic reticulum, and presumably is exported to the apoplast. The mature peptide is 54 AAs long and it has a predicted MW of 5,777 Da. Its exact three-dimensional structure and mechanism of action is as of yet to be determined. Synthetically produced Aracin1 was shown to have an IC_{50} for plant pathogenic

fungi as low as 740ng mL^{-1} and *Arabidopsis thaliana* (*A. thaliana*) overexpressing Aracin1 had reduced infection to the necrotrophic fungal pathogens *Botrytis cinerea* and *Alternaria brassica* (Neukermans, Inze et al. 2015).

Zeamatin

The largest AMP evaluated in this work is originally found at high concentrations in the seed of corn (*Zea mays*) and is called zeamatin (Roberts and Selitrennikoff 1990). It belongs to the pathogen-related, thaumatin-like family of proteins. It is a 227 AA preprotein with a 20 AA export signal sequence at it's N-terminus. Its three-dimensional structure has been resolved and contains two major regions, one made up of α-helixes and the other made up of β-sheets. It is naturally found as an asymmetrical dimer. The three dimensional structure of each monomer is stabilized by 8 disulfide bonds (Batalia, Monzingo et al. 1996). The calculated molecular weight of the mature monomer protein is 22.1 kDa. It is believed that zeamatin acts by permeabilizing fungal membranes (Roberts and Selitrennikoff 1990). As for its antifungal activity, zeamatin was shown to inhibit the growth of *Trichoderma reesei*, *Neurospora crassa*, and *Candida albicans* in liquid culture at a concentration as low as 5µg mL^{-1}. Zone-of-inhibition activity on solid media for these fungi was unique in that it required the addition of sub-inhibitory concentrations of the antifungal nikkomycin Z (Roberts and Selitrennikoff 1990). Zeamatin has also been shown to work synergistically with nikkomycin Z against at least one human fungal pathogen (Stevens, Calderon et al. 2002). Another group showed zeamatin purified from corn to have antifungal activity against *Aspergillus flavus* (Guo, Chen et al. 1997). As for heterologous zeamatin production, zeamatin has been produced as an exported fusion protein in *Neurospora crassa* (Rasmussen-Wilson, Palas et al. 1997). As zeamatin is a natural product of corn and there have

been no reported cases of allergens to this AMP, it is assumed that its overexpression in plants is safe from human consumption.

Comparison of recombinant AMPs to native AMPs

Although overexpressing native AMPs in plants is one method for increasing resistance to pathogens, introducing AMPs that would have a novel mechanism of resistance to a system is of particular interest in that it is more likely to be durable. This is because pathogens will have had less time to evolve ways for overcoming a novel mechanism compared to those that they have already encountered. A proteins functionality is ultimately determined by its AA sequence, therefore the novelty of transgenes used in this research was evaluated by comparing their sequence to known soybean and wheat AMPs and BLAST best matches against these species (see Figure 2.4 and Figure 2.5 for alignments). Of the transgenes used in this research, the plant AMP zeamatin had the highest sequence similarity to both soybean (bit score 301 (Graham, Burkhart et al. 1992)) and wheat (bit score 357) thaumatin-like proteins. This is not surprising as zeamatin was previously predicted to belong to the thaumatin-like protein family. Another plant derived AMP evaluated in this research, Ace, had high sequence similarity to a lipid transfer protein (LTP) of wheat (bit score 110 (Neumann, Condron et al. 1994)) and a predicted LTP of soybean (bit score 132); however, Ace does not transfer lipids across membranes as other non-specific lipid transfer proteins do (Cammue, Thevissen et al. 1995). Surprisingly, no major sequence similarities (bit score < 100) were found with the other plant CDSs used in this study, Aracin1 or gamma-thionin, but they do show some conservation in cysteine residues of soy and wheat thaumatins-like proteins. CDSs coming from animal sources, juruin and drosomycin, had very low sequence similarity compared to soybean and wheat AMPs/BLAST hits. The highest similarity for the two CDSs was between drosomycin and a wheat defensin (bit score 36.3). This

is not surprising as drosomycin is classified as a defensin. Bit scores for comparisons were generated with PSI-BLAST, being iterated until search convergence (Altschul, Madden et al. 1997). Databases were generated from the AMP database CAMPr3 and highest BLASTP hits for each transgene from soybean and wheat genomes. The goal of this research was challenge wheat expressing these recombinant genes with several phytopathogenic fungi to check for increased fungal resistance.

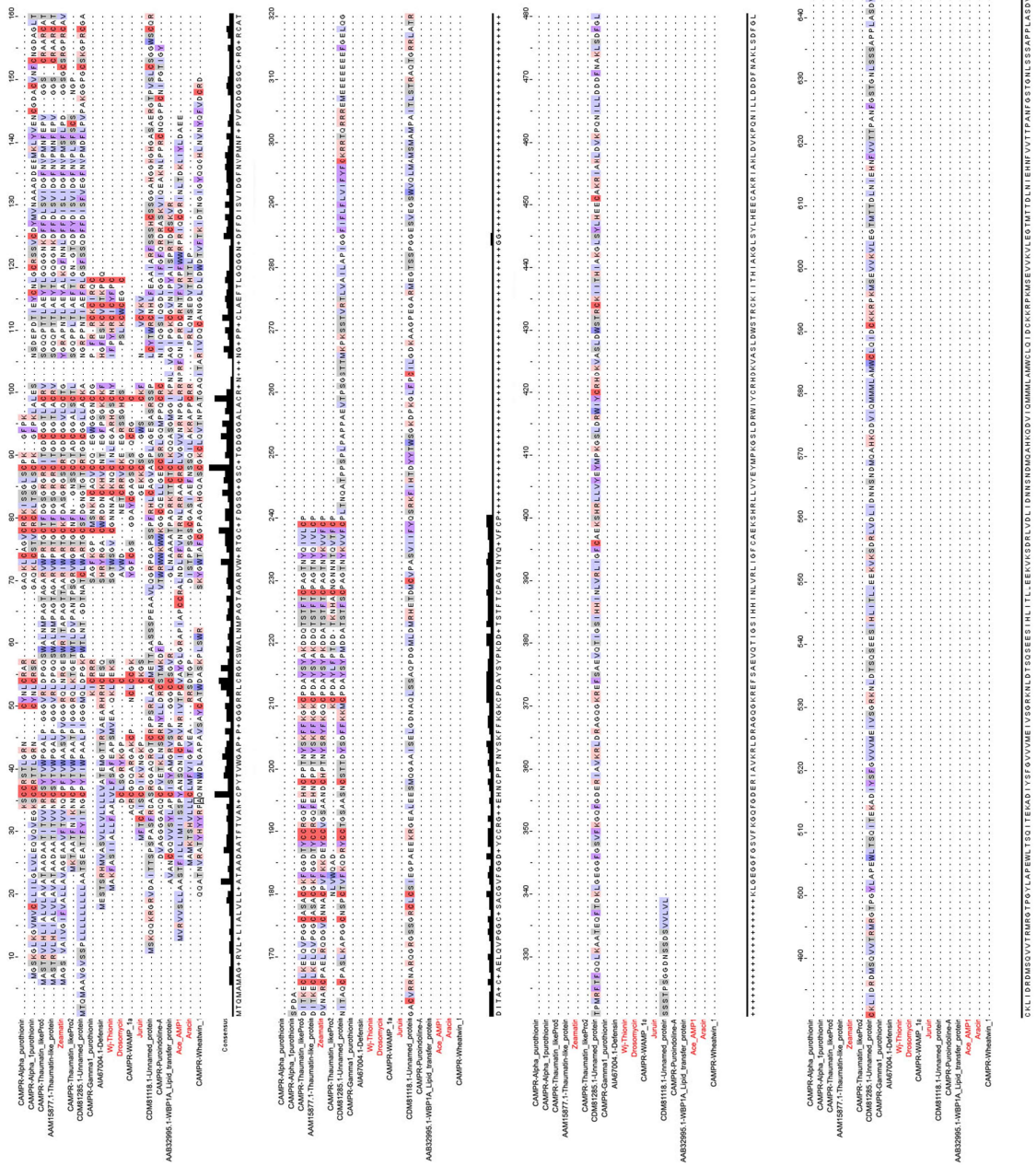


Figure 2.4 Amino acid sequence alignment of transgenes and representative native wheat AMPs and proteins with high sequence similarity.

ClustalX AA alignment of transgenes (labels red) used in this work against high similarity wheat BLAST matches and representative wheat AMPs. Highly redundant AMPs were removed from alignment to help convey information. Cysteine residues are shown in red, residues with similar properties shown highlighted in the same color. Histogram below alignment shows percent residue consensus.

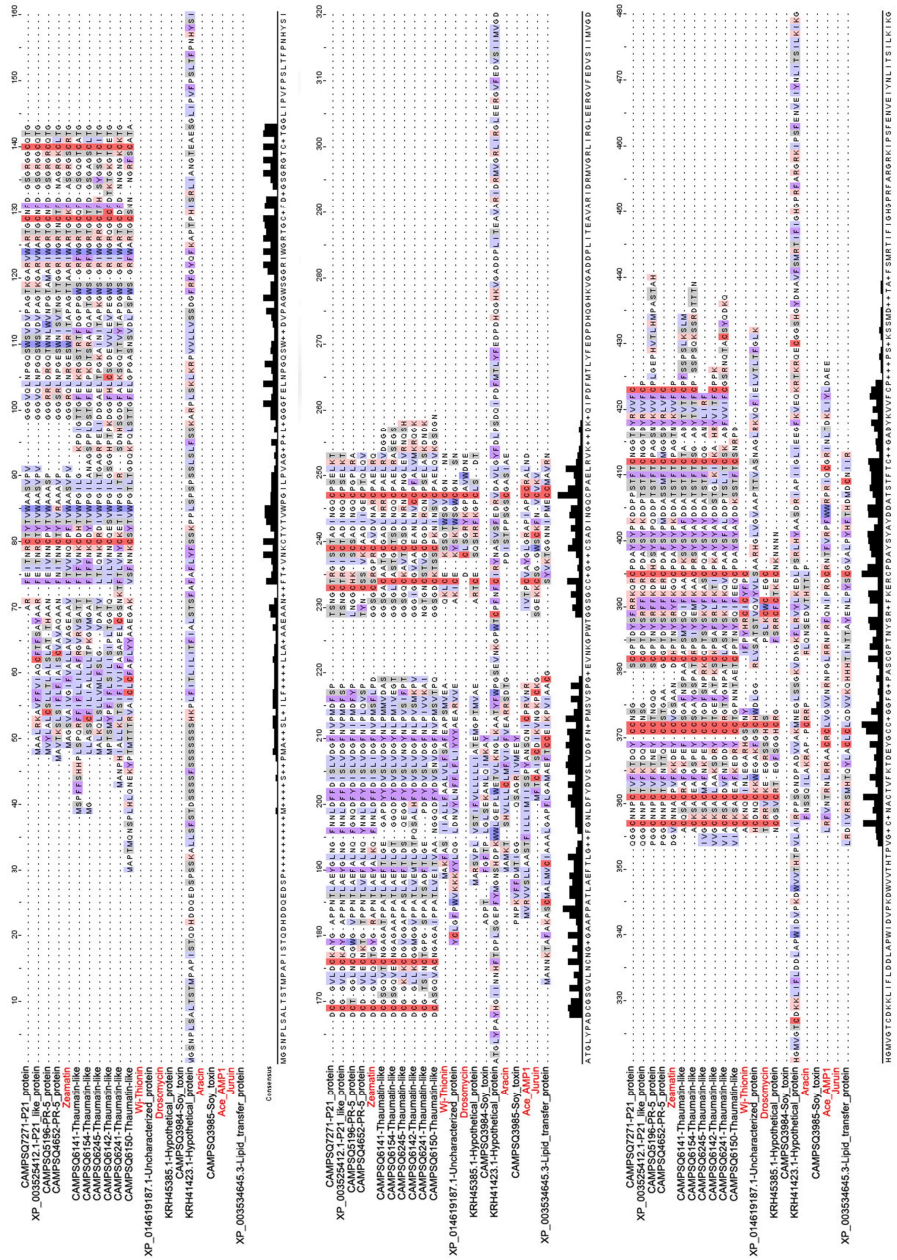


Figure 2.5 Amino acid sequence alignment of transgenes and representative native soybean AMPs and proteins with high sequence similarity.

ClustalX AA alignment of transgenes (labels red) used in this work against high similarity soybean BLAST matches and representative soybean AMPs. Highly redundant AMPs were removed from alignment to help convey information. Cysteine residues are shown in red, residues with similar properties shown highlighted in the same color. Histogram below alignment shows percent residue consensus.

Materials and methods

Overexpression of transgenes in *A. triticum* for the defensin work was accomplished by subcloning protein coding sequences (CDSs) into the pAHC17 expression vector developed by Christensen et al (1996). A diagram of pAHC17 with features of interest are shown in Figure 2.6. CDSs were sub-cloned into this vector via *Bam*HI digestion and directionally confirmed with GOI and vector specific primers found in Appendix C. General subcloning procedures can be found in Chapter 4. Constitutive expression of transgenes is driven by a *Zea mays* ubiquitin (*Ubi-1*) promoter and terminated by an *Agrobacterium* nopaline synthase terminator (Tnos). For better expression of inserted genes, the 5' UTR exon and first intron of *Ubi-1* are directly downstream of the *Ubi-1* promoter. Inclusion of a β-lactamase gene (*bla*) on pAHC17 confers ampicillin resistance for positive selection of this plasmid in bacteria. For transformation of *A. triticum*, pAHC17 plasmids containing GOI's were co-bombarded with pAHC20. pAHC20 is a plant expression vector derived from the insertion of the BAR gene into pAHC17 and confers resistance to the herbicide glufosinate. pAHC17 and pAHC20 are approximately 4.9 and 5.4 kb respectively (Christensen and Quail 1996).

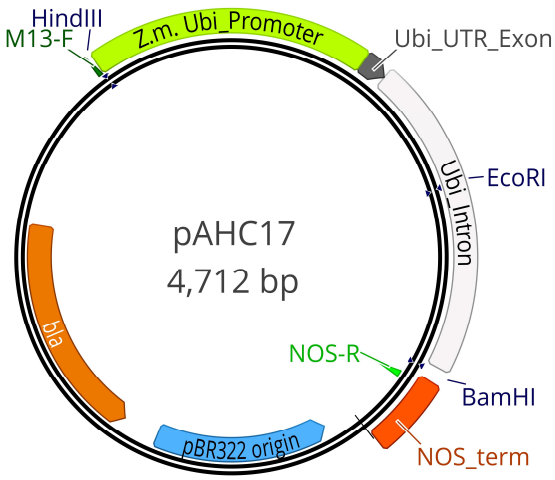


Figure 2.6 Diagram for pAHC17 plasmid

Annotations include *Zea mays* ubiquitin promoter (Z.m. Ubi_Promoter), ubiquitin untranslated region exon (Ubi_UTR_Exon), ubiquitin intron (Ubi_Intron), nopaline synthase terminator (NOS_term), ampicillin resistance gene (*bla*), pBR322 origin of replication, M13 forward (M13-F) and NOS reverse (NOS-R) primer binding sites as well as unique restriction sites.

After subcloning juruin and drosomycin CDSs into pAHC17, plasmids were used for biolistic transformation of wheat calli. Biolistic transformation and tissue culture regeneration of wheat in this research was conducted as described by Tian et al (2019). T_1 through T_3 plants were used for the bioassays described below. Wheat expressing zeamatin, Ace, aracin1, and gamma-thionin CDSs were developed and kindly provided by Monica Navia-Urrutia, Kansas State University, Department of Plant Pathology.

Common bunt bioassays

For common bunt bioassays wheat seed was added to a sterile beaker containing *T. laevis* teliospores, 1g per 100 seed or equivalent, and gently agitated until spores evenly covered seed. The initial inoculum for this research obtained from Dr. William Bockus, Kansas State University, is a Kansas isolate. Transgenic and control seed were sown 4-7cm deep in 66 mL Ray Leach Cone-tainers (Stuewe & Sons; Tangent OR) with potting soil in a randomized

complete block design (RCBD) and placed in a growth chamber at 5-10°C with a 16 hr light /8 hr dark photoperiod. After the second leaf became fully expanded, plants were repotted into 11x14 ø cm pots and returned to growth chambers with light/dark temperatures increased to 20-22/15-18°C respectively. Wheat was rated for presence or absence of teliospores by physically destroying central florets between thumb and forefinger at the soft dough stage. A darkened dough indicated the presence of teliospores (Figure 2.7). Due to its obligate parasite lifestyle, inoculum was maintained by collecting teliospores from bunted Bobwhite wheat. Plants used in experiments were confirmed to express recombinant AMPs through RT-PCR. The mean sample size for this experiment was nine plants and ranged from 5-13 per line. Lines tested include drosomycin lines 6929 & 6933, juruin lines 7079 & 7411, aracin1 lines 8457 & 8524, gamma-thionin line 8556, Ace line 8946, and zeamatin line 8796. All experimental wheat lines were T_3 generation except for Ace and zeamatin lines which were T_2. Plants not expressing transgenes were treated as the tissue culture control and the Bobwhite background was used as a negative control.

Figure 2.7 Bunted wheat seed

Cross-section of wheat seed at dough stage from *T. laevis* infected (A) and non-infected (B) plants.

Fusarium head blight bioassays

F. graminearum used for growth chamber bioassays in the this study was the DON producing strain GZ3639 isolated by Dr. Robert L. Bowden (Proctor, Hohn et al. 1995). For inoculation, a floret undergoing anthesis at a central spikelet was inoculated with 10µL of 100 conidia μL^{-1} solution with a glass syringe. Inoculum was kindly maintained and provided by Guihua Bai's lab, Kansas State University. Plants were grown under a 16 hr light/8 hr dark cycle at 20-22°C/15-18°C respectively in Conviron (Winnipeg, MB) growth chambers. Heads were placed in plastic sandwich bags saturated with moisture for 48hrs for favorable infection conditions. Heads were scored for percent infection at 7, 9, and 12-days post inoculation (dpi). A RCBD was used for each experiment, and was generated by SAS software. Inoculations were performed as flowering facilitated. Only plants expressing transgenes were used for statistical analysis. The first FHB bioassay used drosomycin lines 6929 and 6933 as well as juruin lines 7079, 7398, 7398, and 7411. All experimental lines for bioassay one were T_2 generation. Plants not expressing transgenes were used as the tissue culture control and Bobwhite was used as the negative control. Experimental lines for the second FHB bioassay included drosomycin lines 6929 (T_3), 6933 (T_3), 7778 (T_2), and 8531 (T_2) as well as juruin lines 7079 (T_3), and 8410 (T_2). Control treatments were similar to that of the first FHB bioassay

Tan spot bioassays

TS bioassays were performed as performed by Kim et al. (2012) with modifications. The Kansas isolate FH-86 was used in both bioassays due to its high yield of conidia under *in vitro* growth conditions. Hyphal growth of fungi was perpetuated on ¼ potato dextrose agar (PDA) by inoculating plates and incubating in the dark for three days at 21°C. For inoculum production, V8 agar was inoculated with ¼ PDA culture and incubated in the dark for five days at 21°C.

Hyphal growth was then knocked down with a sterile bent glass rod. Plates were then closed with parafilm and holes were poked in the parafilm with sterile scalpel for added ventilation. Plates were incubated for 5-8 days at a 12 hr light/12 hr dark photoperiod at 21/16°C respectively. Concentric growth is indicative of conidia production. Conidia were harvested by adding 5 mL of sterile distilled water to the petri dish and the mycelial surface was gently rubbed with a sterile spatula. Large debris was removed by passing conidial solution through two layers of sterile cheesecloth. Spore concentration was determined with a Hausser Scientific (Horsham, Pa) Bright-Line improved Neubauer hemacytometer. Conidia concentration was adjusted to 1e4 mL^{-1} and 35 mL of this solution was applied uniformly to 96, 1 month-old plants with a DeVilbiss (Port Washington, NY) atomizer at 10 psi. Leaves were allowed to dry and then plants were placed in a Percival (Perry, IA) I-36DL mist chamber for 48 hr at 20-28°C. At the time of planting, seed was sown into 66 mL Ray Leach Cone-tainers (Stuewe & Sons) with potting soil and placed in a 10x20 rack in a completely random design (CRD) fashion. Plants were rated at 5, 10, 15, and 20 dpi according to the disease rating matrix in Figure 2.8. The two most prominent leaves at time of inoculation, resulting in the worst disease severity, were rated for each plant. The youngest leaf, closest to the apical meristem, is considered leaf one while the next youngest below is termed leaf two. For experiment two the largest lesion per plant was also recorded at 15 and 20 dpi. Plants for experiment one were grown under greenhouse conditions with day/night temperatures of 20-22°C/15-18°C respectively. Plants for experiment two were grown in a Conviron growth chamber under a 16 hr light/8 hr dark cycle at 20-22°C/15-18°C respectively and moved to greenhouse conditions after three weeks, with the same day/night temperatures.

1 – No detectable symptoms

2 – 0-1%

3 – 1-5%

4 – 5-10%

5 – 10-25%

6 – 25-50%

7 – 50-75%

8 – 75-100%

Figure 2.8 Disease rating matrix for Tan Spot bioassays with examples

Plants in tan spot bioassays were given one of the following ratings for disease severity depending on the percentage of chlorosis/necrosis on first and second leaves. Representative wheat leaves from tan spot bioassay one with leaf A scoring 3, leaf B scoring 6, and leaf C scoring 8

Plants were confirmed to express transgenes via RT-PCR. Plants not expressing transgenes were removed from statistical analysis or used as a tissue culture control. The progenitor variety Bobwhite was used a negative control. For the first TS bioassay experimental lines included Ace 8946, aracin1 8524 & 8457, dros 7778 & 8531, juruin 7079 & 8763, gamma-thionin 8556, and zeamatin 8796. Lines 8946, 8763, and 8796 were T_2 generation and the remaining experimental lines were of T_3 generation. For the second TS bioassay experimental lines used included two Ace 8946 T_2, aracin1 8457 T_2 & T_3 & two 8524 T_3, drosomycin 6929 T_2 & 6933 T_2 & T_3, two juruin 7079 T_2 & one T_3, two 7398 T_2, 7411 T_2 & T_3, 8763 T_2, two gamma-thionin 8556 T_3, and two zeamatin 8796 T_2 lines.

Quantitative expression analysis of AMP wheat lines

Several AMP wheat lines used in fungal bioassays were characterized for relative expression levels of transgenes via real-time quantitative polymerase chain reaction (RT-qPCR). This was done by determining arbitrary expression units by normalizing transgenes against the geometric mean of most stable reference genes as described by Fletcher et al (2014). Briefly, controls for generating standard curves were made for reference and transgenes by PCR of appropriate cDNA samples. Reference genes evaluated included *actin*, *cyclophilin*, *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*), and *elongation factor alpha* (*EFα*). Primers used for these genes are the same as those used by Yadav et al. (2015). Juruin and drosomycin primer pair one and qPCR_zeaM-F x ZeaM-R primer pair were used to amplify their respective genes for this work (other unique primer pairs can be derived from Appendix C). Following PCR, amplicons were purified via Qiagen (Hilden, Ger.) QIAquick PCR purification kit. Accurate quantification of DNA was then measured with an Invitrogen (Irvine, CA) Qubit fluorometer using the high sensitivity dsDNA assay kit. Equation 2.0 was used to make 200 µL of 1e9 amplicon μL^{-1} stock solution.

Equation 2.0: $v_i = (\mu L\ (x\ ng)^{-1})\ (1e9\ ng\ g^{-1})\ (((y\ bp)\ (607.5\ g) + 157.9\ g)\ (moles)^{-1})\ (3.32e\text{-}13)$

Where v_i is the initial volume of PCR amplicon to be diluted to 200 µL, the first term is the inverse concentration of the purified amplicon, and y is the bp length of the amplicon. A dilution series encompassing six orders of magnitude (1e1 to 1e6 copies μL^{-1}) was generated for each reference and transgene. The appropriate dilution series were ran with unknown samples for each RT-qPCR run to determine starting copy quantity that could be compared across RT-qPCR

runs. cDNA samples were diluted 1:20 by adding 3µL to 57µL RNase-free H_2O. Real time reactions consisted of 5µL iQ SYBR Green I supermix (Bio-Rad; Hercules, CA), 1.2µL 4µM forward and reverse primer, 2µL sample, and 0.6µL mQ H_2O. RT-qPCR was carried out on a CFX96 Touch real-time PCR detection system (Bio-Rad). Cycle conditions consisted of an initial 3 min, 95°C denaturing and enzyme activation step followed by 40 cycles of 10 sec, 95°C denaturing and 30 sec annealing and amplification at the primer specific T_m. For each RT-qPCR run, dilution series, unknowns, and no-template control were ran as triplicate technical replicates. Starting quantity (SQ) data for *cyclophilin*, *GAPDH*, and *EFα* were loaded into the VBA Microsoft Excel applet geNorm V3.5 freeware for determining the two most stable reference genes (Vandesompele, De Preter et al. 2002). Due to non-specific amplification present in actin reactions, these primers were not used in gene expression analysis. SQ data for GOI's were divided by normalization factors produced by geNorm to give arbitrary expression units to be compared between lines.

Recombinant protein production

Synthetic CDSs for juruin and drosomycin from the pJB_def1 plasmid were subcloned into the bacterial expression vector pLic-MBP-APETx2 (Figure 2.9) (Anangi, Rash et al. 2012). This was done by the addition of a *Kpn*I restriction site and TEV recognition sequence to the 5' end and a *Sac*I restriction site to the 3' end of each gene via PCR primer modification. After subcloning and sequence confirmation (see general methods) plasmids were transformed into Singles BL21 (DE3) *E. coli* produced by Novagen (Burlington, MA). For overnight cultures, fresh streak plates were used to inoculate 5 mL LB media with a final concentration of ampicillin at 100 µg mL^{-1}. Inoculated media was incubated overnight on an orbital shaker at 200 rpm, 37°C. One to two milliliters of overnight culture was used to inoculate 200 mL LB media, with the

same concentration ampicillin, in a 1 L baffled-bottom Erlenmeyer flask. Cultures were incubated on an orbital shaker at 200 rpm, 37°C, until an OD_{600} of about 0.4 was reached. At this point isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to a final concentration of 20 µM to induce expression of recombinant AMPs and temperature was reduced to 30°C to reduce precipitation of these proteins. Recombinant proteins were harvested from *E. coli* via a periplasmic osmotic cold shock method developed by Neu and Heppel (1965). After isolation, the protease inhibitor phenylmethylsulfonyl fluoride (PMSF) was added to a final concentration of 1 mM and samples were filtered through a 0.22 micron filter.

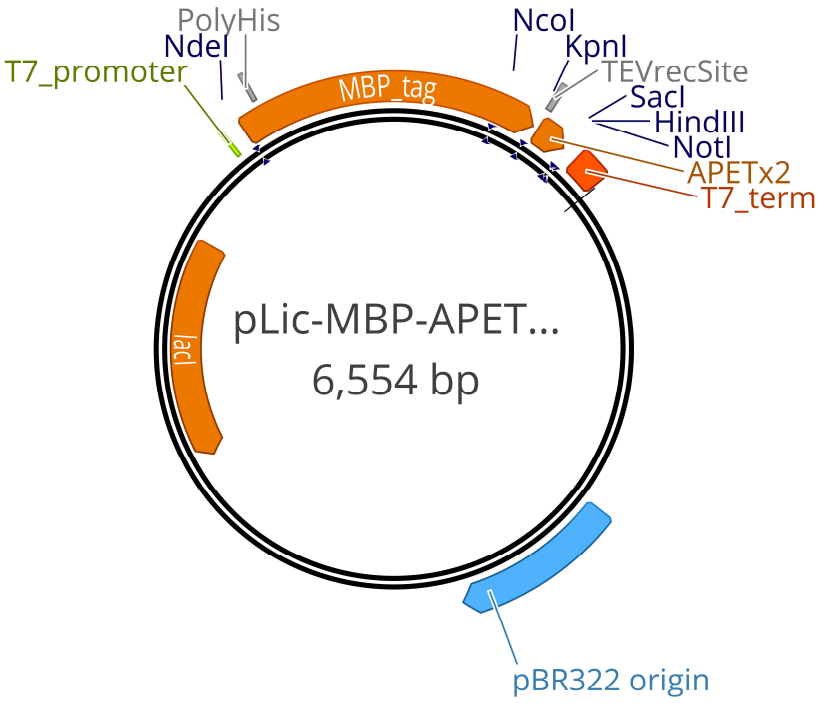


Figure 2.9 Diagram of pLic-MBP-APETx2 plasmid

Annotations include T7 phage promoter (T7_promoter), 6x polyhistidine-tag (PolyHis), maltose binding protein fusion tag (MBP_tag), tobacco etch virus protease recognition site (TEVrecSite), sea anemone ion channel toxin (APETx2), T7 phage terminator (T7_term), pBR322 origin of replication, ampicillin resistance gene (*lacI*), and unique restriction sites.

For protein concentration quantification, a Nanodrop ND-1000 spectrophotometer (Thermofisher: Waltham, MA), software ND-1000 V3.8.1, was used to measure A_{595} using a Coomassie Brilliant Blue G-250 based Bradford reagent and bovine serum albumin (BSA) to generate standard curves. Four dilutions of BSA encompassing 166.7-3,333 μg mL^{-1} were used to generate standard curves for Bradford assays and four dilutions of BSA encompassing 5-100 μg mL^{-1} were used to generate standard curves for microassays. Three repeated measures were taken for each standard. R-squared values for standard curves typically exceeded 0.95. For standard assays 1.5 mL of Bradford reagent (0.005% Coomassie Brilliant Blue G-250 (w/v), 8.5% H_3PO_4, 5% methanol) was added to 30 μL sample and for micro assays 30 μL Bradford reagent was added to 30 μL sample.

Fusion proteins were purified from raw extracts via immobilized metal affinity chromatography (IMAC). This was done with a Ni-NTA purification system (ThermoFisher) under native purification conditions as per manufacturers recommendations. About 14 mg raw protein extract was used per 2 mL Ni-NTA beads. All bound protein from extracts used in this research typically eluted after five 1 mL volumes of native elution buffer, and so, seven 1 mL elution fractions were collected and analyzed per sample.

To remove the MBP fusion tags from recombinant AMPs, TEV protease (TEVp) was used to cleave the TEV recognition sequence engineered between the N-terminal MBP fusion tag and the POI. Successful cleavage would result in a native protein with an N-terminal glycine scar. Cleavage of fusion proteins was initially done with ThermoFisher AcTEV and reactions were set up per manufacturers recommendations. Because disulfide bridge formations for these AMPs are important to their activity, TEV cleavage was analyzed under reducing and non-reducing conditions. For non-reducing conditions, reactions were set up without DTT, and

glutathione (GSH) and glutathione disulfide (GSSG) were added to final concentrations of 607 μM and 413 μM respectively.

Following fusion tag removal, AMPs were separated from TEVp and the MBP fusion tag with a serious of size exclusion filters. For this samples were added to 10 kDa cutoff Millipore-Sigma (Burlington, MA) Amicon filters and centrifuging in an Eppendorf (Hauppauge, NY) 5810-R swing bucket rotor at 4 k·*g*, RT, for 20-30 min or until entire sample was passed through the filter. At least two initial volumes of ddH_2O was added to the 10 kDa retention and centrifuged as previously described to scavenge POI. The 10 kDa passthrough was concentrated with a 3 kDa cutoff Amicon filter by centrifuging as previously described.

Above procedures were confirmed via SDS-PAGE analysis. For this, samples were run on tris-tricine 16.5% SDS-PAGE pre-cast gels produced by Bio-Rad (Hercules, CA) for high resolving power of low MW proteins. Before loading into gels, samples were prepared by mixing 7.5 μL sample with an equal volume of sample buffer (200 mM Tris-HCl, pH6.8, 2% SDS, 40% glycerol, 0.04% Coomassie Brilliant Blue G-250, and 100 mM DTT (added fresh)) and incubating for 10 min at 70°C. Gels were run on a Mini-PROTEAN® gel system (Bio-Rad) at 100 V for 100 min or until the sample buffer dye front reached the bottom of the gel. Anode and cathode buffers were the same running buffer (100 mM Tris, 100 mM Tricine, 0.1% SDS). Gels were stained by placing them in a series of solutions at RT agitated at low speed by a platform shaker. Firstly, gels were fixed in fixative solution (40% methanol, 10% acetic acid, 50% ddH_2O) for 30 min. Gels were then stained with staining solution (0.025% (w/v) Coomassie Brilliant Blue G-250 and 10% acetic acid) for 1 hr. Lastly, gels were destained with three changes of 10% acetic acid for 15 min each.

For more sensitive detection of small proteins on SDS-PAGE gels, a silver staining method was employed. Washes and incubations for silver staining took place in a series of solution baths as described above. Gels were first incubated in fixing solution (40% methanol and 10% acetic acid) for 45 min. Next, gels were washed with ddH_2O twice for 45 min each. Sensitizing followed by incubation in 0.005% sodium thiosulfate for 30 min. Gels were then incubated in 0.1% silver nitrate for 45 min. Following this, gels were briefly washed with ddH_2O and then placed in developer (0.036% formaldehyde and 2% sodium carbonate, made fresh) for 1-2 min. Development was then stopped by placing gels in 50 mM EDTA for 45 min. Lastly, gels were briefly washed twice with ddH_2O. Because the MW of small peptides like the ones used here are difficult to resolve via SDS-PAGE analysis by either Coomassie or silver based staining, confirmation of intact recombinant protein was confirmed via matrix assisted laser desorption/ionization-time of flight (MALDI-TOF). For this samples were run on a Bruker Daltonics (Billerica, MA) Ultraflex III MALDI TOF/TOF mass spectrometer by Kansas State University Biotechnology/Proteomics Core personnel.

Qualitative analysis of transgenic drosomycin and juruin wheat lines

For protein extraction from wheat, leaves were flash frozen with liquid nitrogen and ground in a pre-chilled mortar and pestle. Following this, ten volumes of protein extraction buffer developed by Bjorksten et al. (20 mM sodium phosphate buffer, pH 7.0; 2 mM EDTA, 10 mM sodium diethyldithiocarbamate, and 2% polyvinylpolypyrrolidone (w/v) final concentration) was added and samples were ground for an additional two minutes (1980). Samples were clarified by centrifuging at 4 k·*g* for 10 min. at 4°C. Protease activity was minimized with the addition of 120 µL of 100 mM PMSF and 30 µL ThermoFisher Halt protease cocktail per 12 mL protein extract. Samples were then passed through a 0.2 micron filter. Following this, size exclusion was

performed with a 15 mL, 10 kDa cutoff Amicon filter. The passthrough was collected in a large centrifuge tube as needed until the entire sample was filtered. The <10 kDa passthrough was then concentrated with a 15 mL 3 kDa cutoff Amicon filter by centrifugation at 4 k·*g* for 10 min at RT with the passthrough being discarded as needed. The resulting 3-10 kDa protein extract (LMPE-low molecular weight protein extract) was used for fungal inhibition assays and mass spec analysis (Figure 2.10).

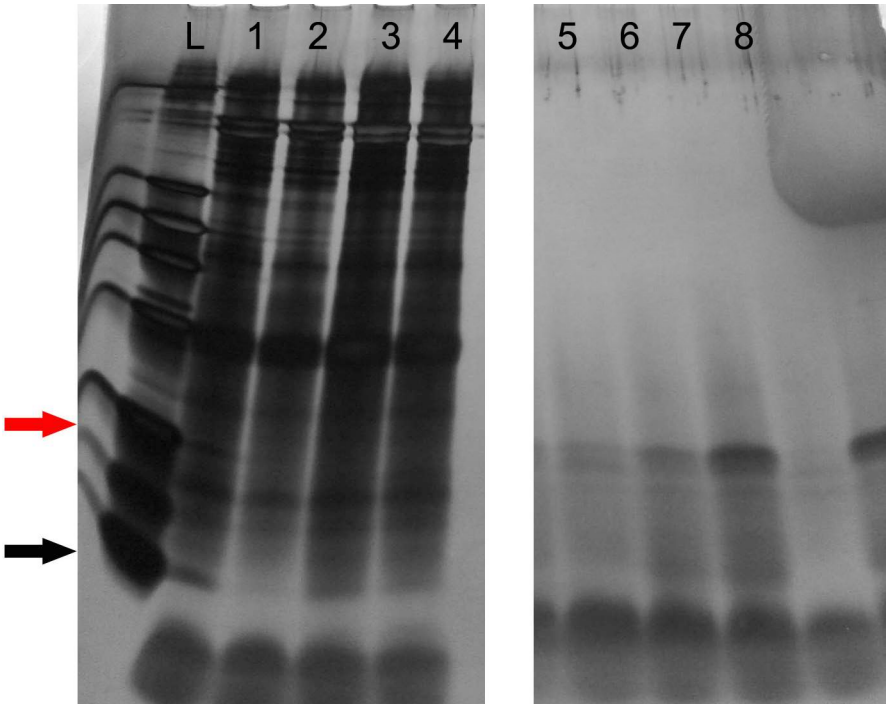


Figure 2.10 SDS-PAGE of wheat lines expressing *juruin* transgenes

Silver staining of SDS-PAGE tricine gel for wheat lines expressing juruin transgenes. Total protein extract of four lines expressing juruin transgene (lanes 1-4) compared to same samples run through 10 kDa cutoff filter (lanes 5-6) against Page Ruler™ low range ladder (L). Two µg total protein and 7.5 µL of <10 kDa for each sample were loaded into wells. Red and black arrows indicate 10 kDa and 3.4 kDa markers respectively. White strip indicates gel splice.

Wheat extracts from the above procedure were tested for inhibiting fungal growth of the filamentous ascomycete *Neurospora crassa* (*N. crassa*) and the generalist phytopathogen

Macrophomina phaseolina (*M. phaseolina*). Strains tested for *in vitro* assays were *N. crassa* FGSC 2489 and *M.phaseolina* isolates 65 (isolated from corn) and 110 (isolated from soybean). For *N. crassa* assays inoculum was produced by incubating a 250 mL flask containing 50 mL PDA with three fungal plugs from a previous culture. The flask was then incubated at 28°C for 5-7 days. Conidia were harvested from flasks by adding 60 mL of phosphate buffer solution containing 0.2% Tween-20 and swirled vigorously for 1 min. The conidial solution was then passed through two layers of sterile cheesecloth to remove large debris. Concentration of conidia was determined with an improved Neubauer 0.1 mm deep hemacytometer (Hausser Scientific; Horsham, PA) and diluted to 5e4 spores mL^{-1}. PDA petri dishes were inoculated by spreading 60 µL of this spore solution onto its surface and air drying for 15-20 min in a biocontainment cabinet.

M. phaseolina inoculum was produced by inoculating foil covered beakers containing ¼ PDB (potato dextrose broth) with three fungal plugs of hyphae perpetuated on ¼ PDA. Beakers were closed and incubated in the dark for 14 days at RT, after which, fungal mats consisting of sclerotia were removed with a sterile spatula and dried by placing on several changes of paper towel. Mats were then dried on paper towels for several days in a laminar flow hood. Fungal mats were then ground to a sclerotial powder in a sterile mortar and pestle. For assays, 800 µL of sclerotial solutioin (1% sclerotia (w/v), 0.015% agarose (w/v), and 100 µg mL^{-1} rifampicin) was spread with a sterile bent glass rod on ¼ PDA plates and allowed to dry for 15-20 min. A second type of *M. phaseolina* assay was performed where a hyphal agar plug was placed in the center of a petri dish and deflection of the hyphal front was read at three and four days post-inoculation.

For these assays six sterile Fisher P5 filter paper discs, reduced in size to 7 mm ø with a hole punch, were placed on the surface of inoculated plates and 8 µL of treatment solution was

added to each disc. A balanced incomplete block design was used for each experiment with three replicates of 12 treatments being split across six blocks. For this a RCBD was generated in R and blocks were split between two subblocks. Each experiment used a 10% glycerol negative control and a 100 μg mL^{-1} nystatin positive control. Two concentrations of LMPE were tested for each experimental treatment, 10 and 30 μg mL^{-1}. *N crassa* plates were incubated in the light at 28°C and a zone of inhibition was checked for 20 hours post inoculation. *M. phaseolina* plates were incubated in the dark at 28°C and read for inhibition after three days. For *N. crassa* conidia and *M. phaseolina* hyphal deflection assays, LMPE from drosomycin 6933 and juruin 7079, 7411 T_3 lines and T_2 juruin line 7398 were evaluated.

For mass spectroscopy (mass spec) analysis of wheat LMPE, peptides were first fractionated via RP-HPLC. This was done using a Beckman Coulter (Brea, CA) System Gold HPLC system using a 126 solvent module and a 168 detector. About 14 μg of sample was used for each analysis. Samples were loaded onto a C18 column with 12 min of 0.1% trifluoroacetic acid (TFA) at 0.5 mL min^{-1} and eluted with a 0-50% acetonitrile gradient against 0.1% aqueous TFA for 80 min at RT at the same flow rate. Elution was monitored spectrophotometrically at 220 and 280 nm. Fractions were collected when absorbance was indicated. Fractions were collected in 1.5 mL microfuge tubes and lyophilized until MALDI-TOF analysis, at which time samples were diluted with 10 μL of ddH_2O and analyzed with a Bruker Ultraflex III.

Fungal Bioassay Results and Discussion

CB bioassay

For the CB bioassay the mean sample size for the experiment was nine plants and ranged from 5-13 plants per line after removing plants not expressing transgenes. Of the plants tested, only two showed no infection, one coming from the aracin1 line 8524 and one from the tissue-

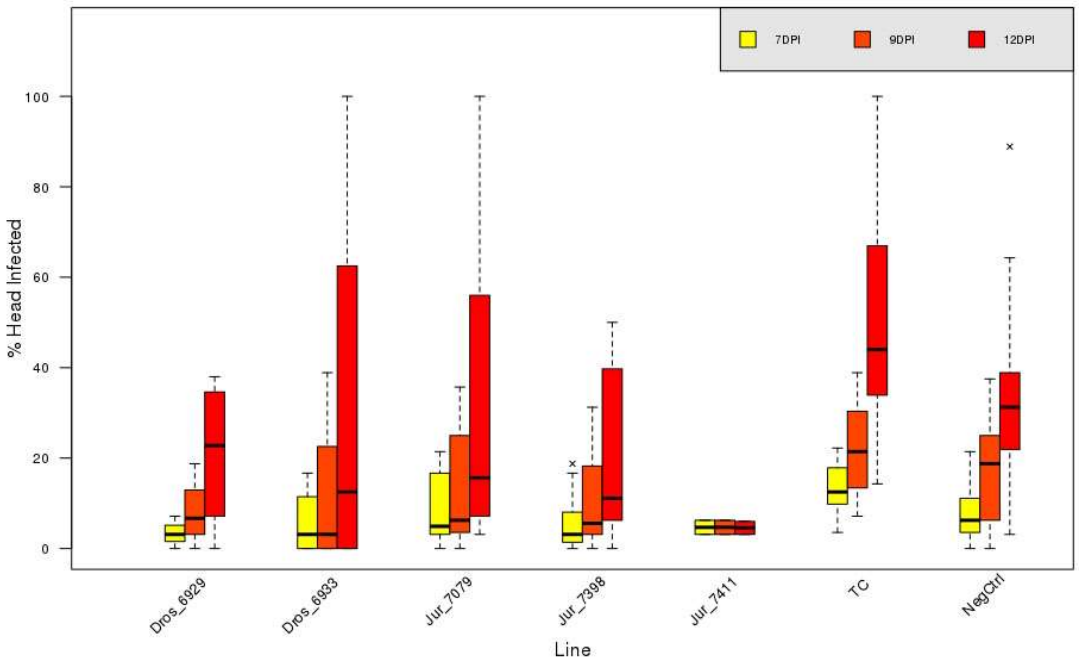
culture control. Seed was collected form the two resistant plants and ten progeny each were challenged with CB to check for resistance. None of these plants showed resistance. Pearson's Chi-square tests showed no significant difference between control treatments and experimental treatments.

Increased resistance to the fungal pathogens used in the bioassays above remains to be seen for the transgenes evaluated here. This is not necessarily surprising for CB bioassays as it is a basidiomycete and there are few examples of AMPs having inhibition activity against this phylum of fungi. This lack of evidence could simply be that AMP activity is typically vetted by well-established *in vitro* assays using ascomycete fungi as opposed to basidiomycetes. Despite this, the importance of basidiomycete pathogens to human and plant health is undeniable. With the large amount of research being done on AMPs it is inevitable that research groups have tested them against these types of pathogens; therefore, lack of evidence for the non-efficacy of AMPs against basidiomycetes could simply be a result of unpublished negative results. On a molecular basis there is evidence that AMPs can inhibit ascomycetes, but not basidiomycetes. There are a number of cases of basidiomycetes that produce thaumatin-like proteins, but not ascomycetes (Liu, Sturrock et al. 2010). Production of this major class of AMP by basidiomycete fungi could indicated an evolutionary adaptation for overcoming their inhibitory effects. Regardless, the CB bioassay was chosen because it is extremely easy to implement. The systemic nature of the fungus at the early stage of plant growth rules out avoidance of recombinant AMPs by the fungus due to tissue specific expression of transgenes. Also, the presence of the fungus throughout the entire lifecycle of wheat could contribute to enhanced susceptibility of the fungus to recombinant AMPs. To increase sensitivity of this bioassay inoculum pressure could be reduced by lowering seed-to-spore mass when inoculating, although this is not a common

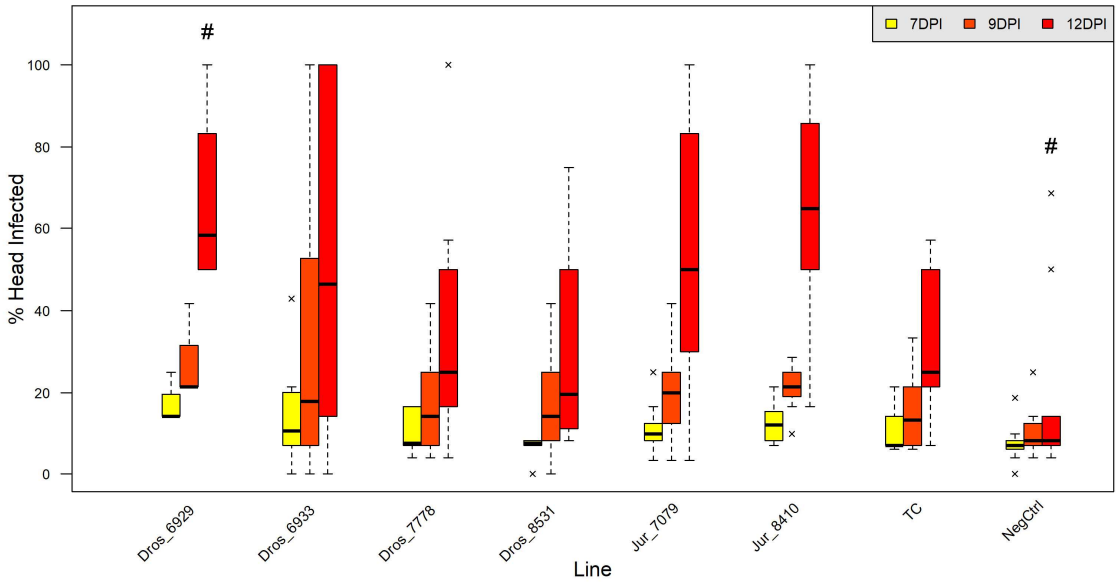
practice (Wilcoxson and Saari 1996, Lu, Gaudet et al. 2005, Fofana, Humphreys et al. 2008). A positive control with an R-gene specific to the race used in this work could be used to exclude the possibility of undue inoculum pressure. Foregoing these possibilities, the transgenes tested here do not appear to increase plant resistance to the phytopathogenic fungus *T. laevis*.

FHB bioassays

For the first FHB bioassay sample size ranged from 3-13 except for Juruin-7411 (sample size two). Although disease severity was generally lower in transgenic plants, a one-way analysis of variances (ANOVA) showed no significant differences (smallest *p* value 0.21 for 7dpi) among treatments for each measurement period and for the area under the disease progression curve (AUDPC) (Figure 2.11 graph A). For the second bioassay sample size ranged from 4-10. ANOVAs for the second bioassay showed significant differences for disease severity at 12dpi and for the AUDPC ($p \leq 0.05$) (Figure 2.11 graph B). A post-hoc Dunnett's multiple comparison tests (Dunnett's test) against negative and tissue culture controls showed a significant difference between the drosomycin line 6929 line and the negative control ($p \leq 0.05$) at 12 dpi. No significant differences were found for AUDPC (smallest *p* value 0.055 for drosomycin line 6933 and negative control)



A



B

Figure 2.11 FHB bioassays one and two

FHB disease severity in percent head infection on transgenic wheat expressing *juruin* and *drosomycin* transgenes at 7, 9, and 12 days post inoculation (DPI) for bioassay one (A) and two (B). Experimental treatments compared against bobwhite background and tissue culture (TC) controls. Columns with #'s indicate significant difference between the two via Dunnett's test ($p < 0.05$). "×" indicate outliers.

To increase the odds of seeing inhibition of fungi from the tested AMP transgenes, the ascomycete fungi *F. graminearum* and Ptr were also evaluated. Like CB bioassays, few differences were seen in FHB and TS bioassays when comparing experimental treatments and control treatments. Both FHB bioassays showed good variability in disease severity indicating the possibility of resolving resistant phenotypes. A conflicting trend between the two bioassays is that experimental treatments seemed to fair better compared to control treatments in bioassay one, but worse in bioassay two. It is possible that this is a result of observer error or possibly a genotype by environment effect for experimental lines; however, the high degree of consistency between TC controls between the two experiments could rule this out. Juruin line 7411 showed good resistance compared to TC and negative controls in FHB bioassay one. The small sample size of two for this line in this bioassay means this line needs to be re-evaluated against FHB as this phenomenon could be due to random chance. Because other juruin lines showed similar disease severity compared to other transgenes and controls tested here, it is likely that any resistance seen from juruin line 7411 is due to some other factor and not expression of the *juruin* transgene itself. Such factors could include native gene knockout during transgene integration and trans influence on expression of other native genes. Similarly, the increased susceptibility of drosomycin line 6929 in the second FHB bioassay is likely due to some other factor rather than expression of the *drosomycin* transgene itself. Considering the low variability of the negative control in this experiment, the significant difference observed against drosomycin 6929 is questionable. Regardless of the mechanism of resistance or susceptibility, these lines need to be evaluated against FHB another time as they could elucidate natural sources of resistance.

TS bioassays

The sample size for TS bioassay one ranged from 4-12. The mean sample size per treatment for experiment one was eight plants. Disease severity was generally worse on experimental groups compared to BW and TC controls for leaf one but not necessarily for leaf two (Figure 2.12). Statistical analysis of response variables was performed with Kruskal-Wallis rank sum difference tests (Kruskal-Wallis test). If a significant difference was found, a post-hoc Dunn multiple comparison test (Dunn test) was performed. *P* values for Dunn tests are reported as Bonferroni adjusted *p* values. A Dunn test showed a significant difference between the drosomycin 8531 line and BW control at 5dpi for leaf one (*p* value = 0.008). No significant differences were found at 10, 15, and 20dpi. Also, no significant differences were found for disease progression or AUDPC for either leaf one or two (smallest *p* value 0.072 for disease progression and 0.076 AUDPC).

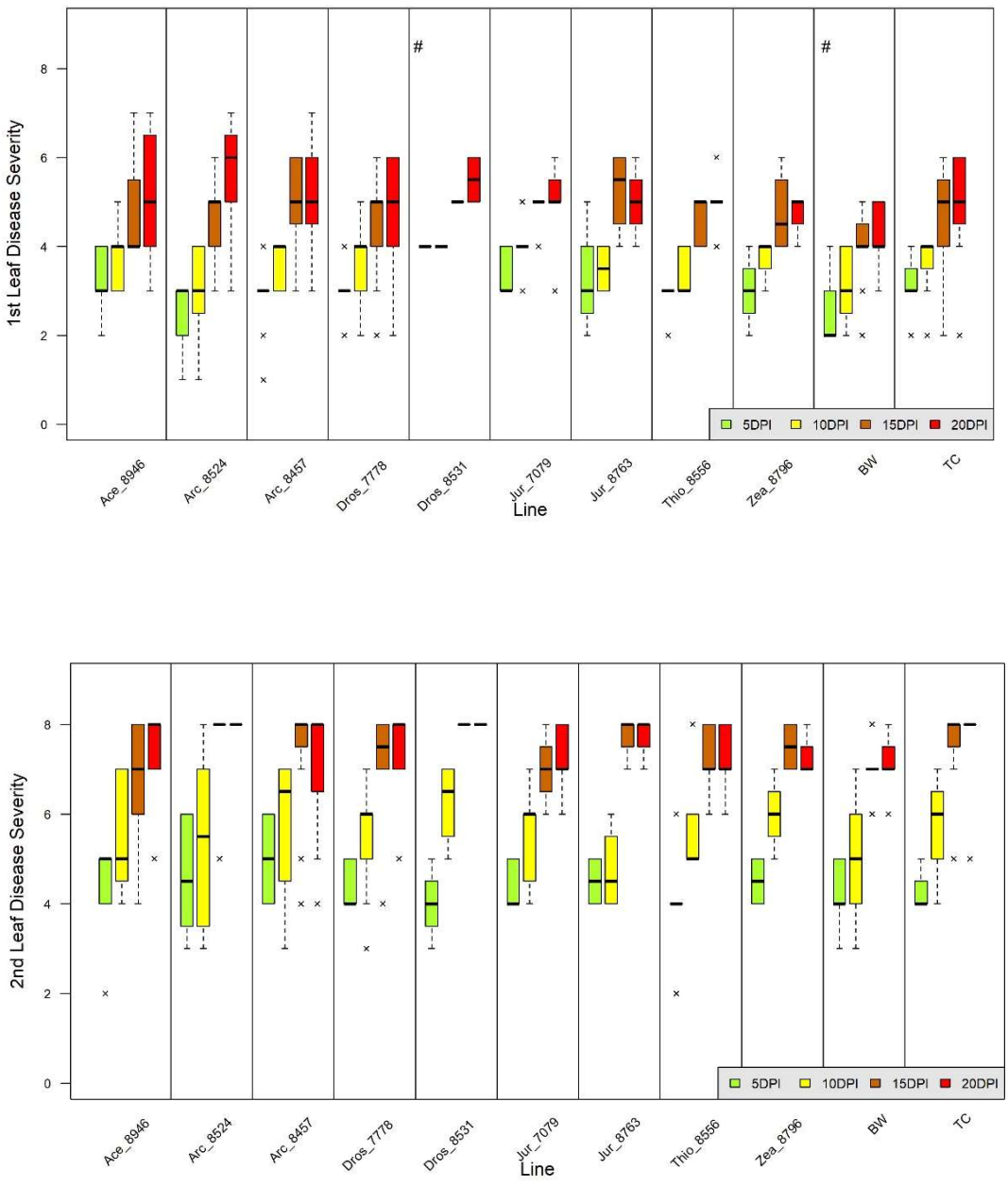


Figure 2.12 TS bioassay one disease severity on first and second leaves

Boxplot for TS bioassay one of disease severity on transgenic wheat lines expressing *Ace-AMP1* (ace), *aracin*1 (arc), *drosomycin* (dros), *juruin* (jur), *gamma-thionin* (thio), and *zeamatin* (zea) compared to tissue culture control (TC) and transformant background line Bobwhite (BW) for first (A) and second leaves (B). Readings were taken at 5, 10, 15, and 20 days post-inoculation (DPI). Severity is rated according to the disease severity matrix from Figure 2.8. Hashes indicate a significant difference ($p < 0.05$) between groups via Dunn-test and "×" indicates outliers.

Sample sizes for TS bioassay two ranged from 3 to 22. The mean sample size was 10 plants per treatment. Disease severity on the second leaf was generally worse compared to first leaf disease severity (Figure 2.13 & Figure 2.14). Like experiment one, experimental treatment means were generally higher for disease severity compared to BW and TC control treatments. Statistical analysis for experiment two was conducted in the same manner as experiment one. A significant difference ($p < 0.05$) was found for the first leaf at 10, 15, and 20 dpi. Post-hoc Dunn tests showed a significant difference at 20 dpi for leaf one between juruin line 7411-B1 and the TC control only. There were no significant differences for disease severity for leaf two, smallest *p* value 0.11. A significant difference was found for disease progression between 5 and 10dpi for the first leaf; however, a Dunn test could not determine a significant difference between treatments (smallest *p* value 0.06). Disease progression was similar at other points among treatments. An ANOVA for largest leaf lesion showed no difference between treatments at 15 or 20 dpi, smallest *p* value 0.39 (Figure 2.15). A significant difference was found in the AUDPC for leaf one, but a post-hoc test resulted in no differences between treatments (smallest *p* value 0.07). A subset of plants for the Ptr experiment two were used for transgene expression analysis. When comparing high expressing outliers to other plants, no increased resistance was observed.

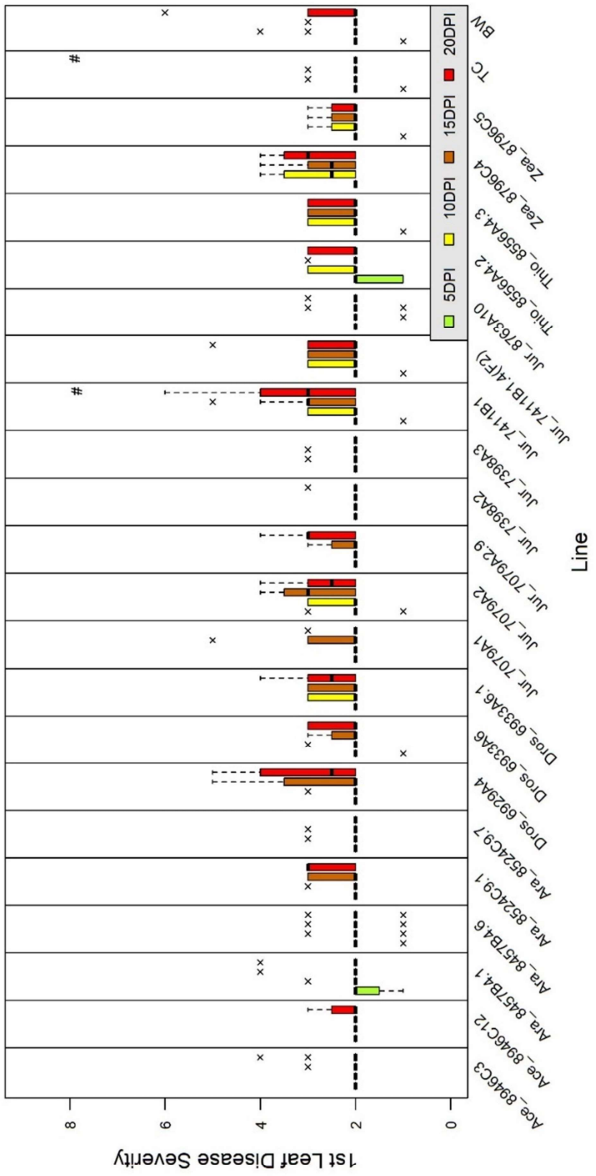


Figure 2.13 TS bioassay two progression of first leaf disease severity

Boxplot for bioassay two of disease severity on transgenic wheat lines expressing *Ace-AMP1* (ace), *aracin*1 (ara), *drosomycin* (dros), *juruin* (jur), *gamma-thionin* (thio), and *zeamatin* (zea) compared to tissue culture control (TC) and transformant background line bobwhite (BW) for first leaves. Readings were taken on the youngest infected leaf at 5, 10, 15, and 20 days post-inoculation (DPI). Severity is rated according to the disease severity matrix from Figure 2.8. Hashes indicate a significant difference ($p < 0.05$) between groups via Dunn-test and "×" indicates outliers.

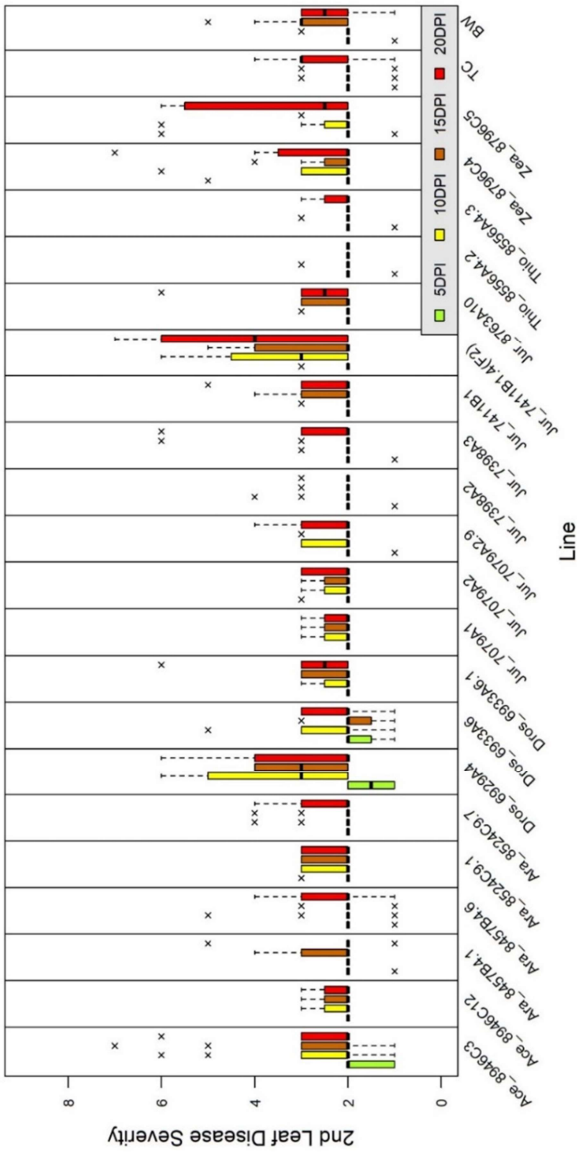


Figure 2.14 TS bioassay two progression of second leaf disease severity

Boxplot for bioassay two of disease severity on transgenic wheat lines expressing *Ace-AMP1* (ace), *aracin*1 (Ara), *drosomycin* (dros), *juruin* (jur), *gamma-thionin* (thio), and *zeamatin* (zea) compared to tissue culture control (TC) and transformant background line bobwhite (BW) for second leaves. Readings were taken on the second youngest infected leaf at 5, 10, 15, and 20 days post-inoculation (DPI). Severity is rated according to the disease severity matrix from Figure 2.8. "×" indicates outliers.

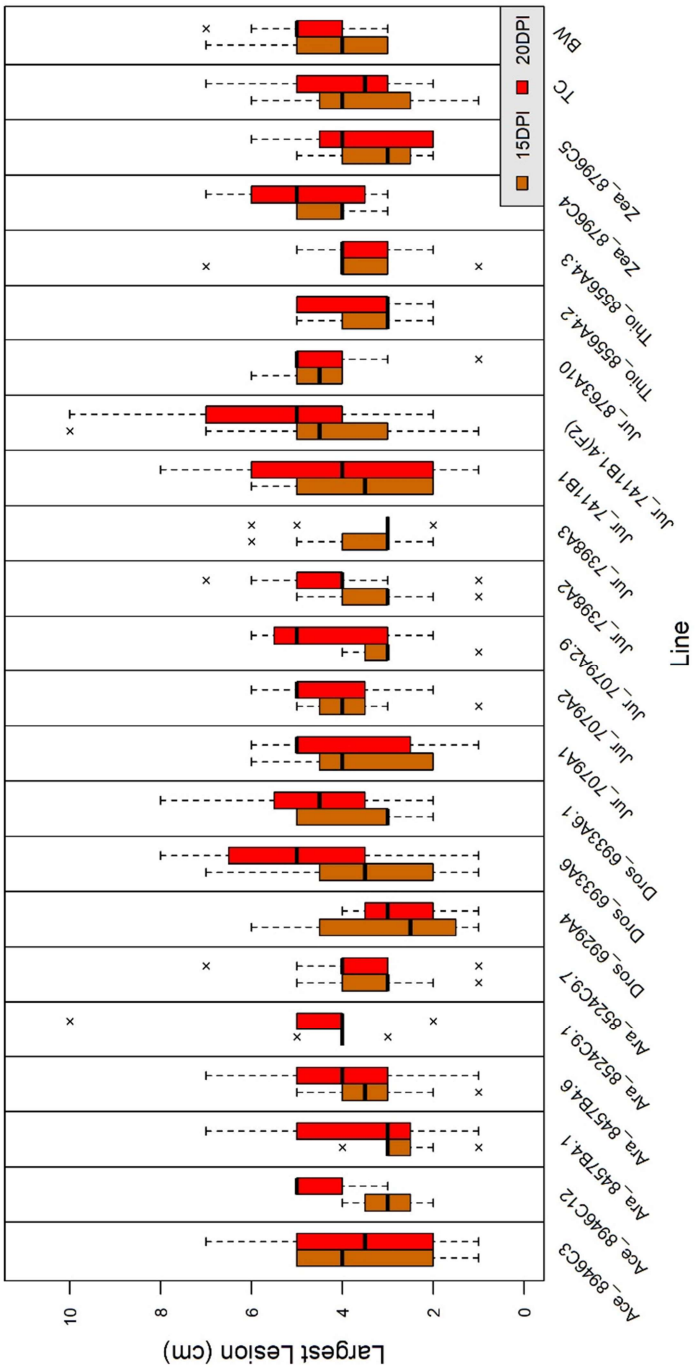


Figure 2.15 TS bioassay two largest leaf lesion

Boxplot for bioassay two of largest lesion size (cm) on transgenic wheat lines expressing *Ace-AMP1* (Ace), *aracin*1 (Ara), *drosomycin* (dros), *juruin* (jur), *gamma-thionin* (thio), and *zeamatin* (zea) compared to tissue culture control (TC) and progenitor line Bobwhite (BW). Readings were taken on first or second leaf that displayed the largest lesion at 15 and 20 days post-inoculation (DPI). "×" indicates outliers.

For the TS bioassays, bioassay one showed a wide range in disease severity while bioassay two did not. Not surprisingly, pooled information from each bioassay showed a significant difference between leaf one and two at each observation point (ANOVA p value < 0.05) between the two experiments. Wide response ranges such as the one found in bioassay one are often desirable for resolving significant differences between treatments. Because both bioassays were conducted at the same time of year, March 2018/2019, it is unlikely that environment played a part in this variation. One factor that could account for differences in disease severity between the two experiments is that numerous plants from bioassay two were diseased with powdery mildew (undetermined species). It is possible that a low level of infection from the fungal agent of this disease could activate systemic acquired resistance in the plants increasing their resistance to TS. If so, development of weakened powdery mildew species for "vaccinating" wheat in TS susceptible areas may be a viable method of TS control. Significant difference between juruin line 7411 and drosomycin line 8531 were detected in these experiments compared to the TC & BW controls respectively (see Figure 2.12.A and Figure 2.13). Since other lines expressing the same transgenes in these experiments did not show an increase in disease severity, it is probable that the increase in disease severity is not due to the expression of the specific transgene, but line specific or simply by random chance. Several reasons for line specific differences has previously been discussed.

Genetic characterization of transgenic wheat

Particle bombardment of wheat calli for generating transgenic wheat resulted in ten and fourteen lines of wheat that were positive for recombinant *juruin* and *drosomycin* respectively. A subset of these lines were used in the above bioassays allowing the genetic characterization of

six drosomycin and seven juruin lines at T_1, five drosomycin and four juruin lines at T_2, and four drosomycin and two juruin lines at the T_3 generation (Table 2.1). For other transgenes one zeamatin & Ace line were characterized at the T_2 generation. Two aracin1, one Ace, and two gamma-thionin lines were genetically characterized at the T_3 generation. For the lines tested across multiple generations, silencing of transgenes happened for one drosomycin line at T_1 and another at the T_2 generation. For the juruin lines transgene silencing happened for two lines at the T_1 generation. Transgene inheritance in progeny ranged from about 50-100% for most of these lines. Single transgene inserts typically have a dominance type segregation ratio of 3:1. Lines here often showed inheritance ratios below this. A number of reasons are possible for distortion in transgene inheritance including reduced transgene transmission and loss of the transgene. These two instances can result from chromosomal instability, deleterious mutations, chimerism, and transgene excision due to multiple inserts (Pawlowski and Somers 1996). Contamination of PCR reactions is highly unlikely as negative controls for all PCR reactions showed no amplification. Furthermore, transgene confirmation for the Ptr bioassay two was completed by an alternative method in a CRD fashion using robotics for genomic DNA (gDNA) isolation and PCR master mix aliquoting. For these line five of the twenty-two lines evaluated had distorted segregation ratios of transgenes below 3:1 (Chi-square $p < 0.05$).

Positive $T_0 \rightarrow T_1$		T_1				T_2				T_3			
GOI	Line	Obs.	% GOI pos.	Exp. Obs.	% Pos. Exp.	Obs.	% GOI pos.	Exp. Obs.	% Pos.Exp.	Obs.	% GOI pos.	Exp. Obs.	% Pos. Exp.
Dros	6459B	19	89.5%	16	93.8%	20	90.0%	16	0.0%				
Dros	6929A	5	40.0%	2	100.0%	51	47.1%	21	100.0%	25	96.0%	22	95.5%
Dros	6933A	7	85.7%	6	83.3%	21	90.5%	18	83.3%	64	67.2%	36	83.3%
Dros	7778A	10	80.0%	8	100.0%	13	100.0%	13	92.3%	10	100.0%	10	100.0%
Dros	8531A	10	80.0%	6	100.0%	16	43.8%	7	71.4%	7	71.4%	4	100.0%
Dros	8631A	8	75.0%	6	0.0%								
Jur	7079A	6	83.3%	5	100.0%	50	72.0%	32	90.6%	60	65.0%	35	100.0%
Jur	7398A	28	78.6%	20	100.0%	54	66.7%	31	93.5%				
Jur	7403A	6	50.0%	3	0.0%								
Jur	7408A	10	90.0%	9	100.0%								
Jur	7411A	7	100.0%	7	100.0%	31	74.2%	19	89.5%	47	100.0%	31	100.0%
Jur	8410C	6	100.0%	6	0.0%								
Jur	8763A	10	60.0%	5	60.0%	37	45.9%	11	100.0%				
										Ara	8457B	77	76.6%	42	100.0%
Zea	8796C					75	57.3%	36	97.2%	Ace	8476B	30	16.7%	2	100.0%
Ace	8946C					71	67.6%	32	87.5%	Ara	8524C	60	98.3%	45	100.0%
										Thio	8538C	30	86.7%	15	6.7%
										Thio	8556A	85	56.5%	35	85.7%

Table 2.1 Wheat segregation and expression information for recombinant wheat lines used in fungal bioassays

Pooled data for recombinant AMP wheat lines. *Abbreviations*: Observation (obs.), expression (exp.), gene of interest (GOI), *juruin* (jur), *drosomycin* (dros), *zeamatin* (zea), *aracin* (ara), *Ace-AMP1* (ace), *gamma-thionin* (thio).

Quantitative expression analysis for AMP wheat lines

Before starting quantification of transgene expression for experimental samples, primer pairs were validated with melt curves and had R^2 values equal to or greater than 0.98. Efficiencies were around 90-110%. Non-specific amplification of actin primers excluded their use from this analysis. It is probable this primer pair was designed from sequence information for the close relative *Aegilops tauschii* without forehand knowledge of the *T. aestivum* ortholog sequence (Primer-BLAST observations). The gene stability measure (M-value) for housekeeping genes *cyclophilin* and *EFα* were the most stable (lowest M-value) for pooled information for each transgene. Mean M-values for each housekeeping gene were EFα = 1.72, *Cyclophilin* = 1.93, and GAPDH = 2.30. With the exception of the drosomycin T_3 line 6933A6.1, transgenes for these lines were expressed at levels of higher than the geometric mean of housekeeping genes *cyclophilin* and *EF-α*. Juruin lines had higher mean expression than other transgenes lines used for quantitative expression analysis (Figure 2.16). The juruin T_3 line 7411 had significantly higher expression than all other transgene lines except for the T_2 drosomycin line 6933 (Tukey HSD adjusted p value < 0.05). Results from this analysis show that transgene expression is generally reduced in subsequent line generations (lines 6933 & 7079), with the exception of line 7411.

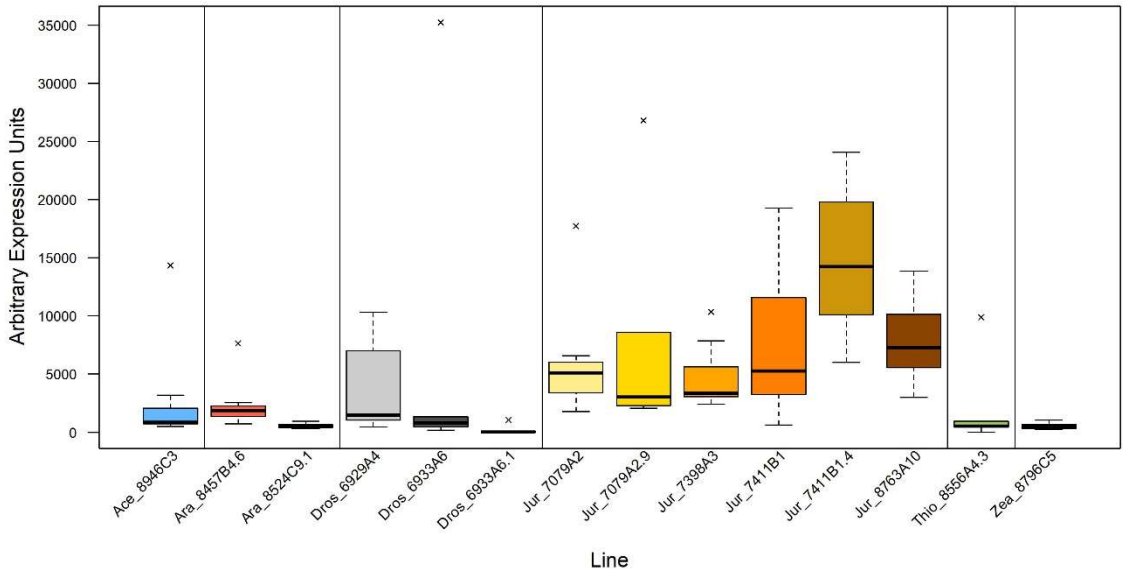


Figure 2.16 Quantitative expression analysis of AMP lines used in fungal bioassays

Arbitrary transgene expression for a subset of transgenic line lines used in fungal bioassays. Line numbers containing a decimal are T3 generation while others are T2 generation. *Abbreviations*: *aracin1* (ara), *drosomycin* (dros), *juruin* (jur), *gamma-thionin* (thio), and *zeamatin* (zea)

Non-efficacy of these transgenes against the pathogens tested here could be due to several factors. One reason for this is plants may not be sequestering recombinant AMPs to where they interact with these pathogens. If efficacy is to be modeled after natural systems, native plant AMPs typically have apoplastic targeting sequences. All transgenes here except for *juruin* have a predicted apoplastic targeting sequence. All the fungi used here come into contact at one point or another with the plants apoplast. It is therefore reasonable to assume that there is interaction between recombinant AMPs and fungi. Predicted targeting sequences of the transgenes or transgenes themselves could be fused to a reporter gene such as GFP to confirm localization of these transgene products. Predicted targeting sequences could also be removed from transgenes to check for a cytoplasmic-derived resistance against these pathogens. The absence of an apoplastic targeting sequence for the *juruin* transgene may be responsible for its

higher expression in wheat. Although expression of these transgenes was confirmed, presence of their protein products was not. This is mostly due to the lack of specific antibodies readily available for these AMPs. One method for overcoming this limitation is to use antibodies developed for closely related proteins (Graham, Burkhart et al. 1992). This could be a viable option for proteins like zeamatin that have high sequence similarity to numerous other plant AMPs (see Figure 2.4 & Figure 2.5). Another approach to identify presence of these recombinant proteins would be to use a qualitative scheme employing a combination of biochemical techniques, an option explored in this research. Non-efficacy could also be due to low recombinant protein yields in wheat as well. Although transgenes from these lines were shown to have high levels of mRNA compared to housekeeping genes, this does not necessarily account for high levels of protein product. For example, several studies in yeast have shown poor correlations ($R^2 \leq 0.17$) between quantitative transcriptome and proteome (Reviewed in: Ingolia, Ghaemmaghami et al. 2009 supp. mtrl.). In addition, outlier plants from the RT-qPCR analysis from this research that were part of TS bioassay two did not show significantly higher resistance compared to other plants of the same line for any response variables. Methods described above for qualitative identification of recombinant proteins could also be used for quantitative analysis. Increasing yields of transgene products in wheat could be investigated further using different promoters to drive expression of transgenes. Another less appealing, but highly probable reason for failure of these recombinant proteins to inhibit these fungal pathogens is simply that the pathogens lay outside the spectrum of inhibitory activity.

Other steps that could be taken to further investigate these transgenes would be to increase the amount of lines evaluated and use several isolates/strains per bioassay. The lines developed here could be used to test for increased resistance to other fungal pathogens of wheat.

The transgenes used here can also be introduced to other plants to check for efficacy against other fungal phytopathogens. For example, one line of soybean with *juruin* and two lines with *drosomycin* were developed for this work but have yet to be characterized. In addition, one line of *A. thaliana* with *juruin* and two lines positive for *aracin1* transgenes have also been developed. These plants have unique fungal pathogens that may lay within the spectrum of inhibition for these AMPs.

Recombinant AMP production from *E. coli* for *in vitro* assays results and discussion

For this research, an attempt was made to produce purified juruin and drosomycin AMPs for *in vitro* assays against fungal phytopathogens. This was started to verify non-efficacy of these AMPs against the pathogens tested in this research and others. *In vitro* assays such as these have several advantages compared to *in planta* assays mainly due to a greater degree of experimental control. For example, incubators for growing fungal cultures typically have less variability in light, temperature, and humidity compared to greenhouse experiments (personal observation). *In vitro* assays typically consist solely of the test species while *in planta* assays can include endophytes and possibly other non-intended pathogens such as fungi and insects. Also, *in vitro* assays with purified recombinant protein offer the ability determine dose dependent inhibition of fungi whereas transgenic plants largely produce uncontrolled levels of recombinant product. Another advantage of this type of assay is AMPs can be produced from microbes such as *E. coli* and tested against fungi in a matter of weeks-to-months compared to month-to-years for some plant systems. In addition to using *E. coli* for recombinant protein production, low molecular

weight proteins were isolated from wheat lines expressing *juruin* and *drosomycin* and tested against several fungi.

Protein extracts from *E. coli juruin* and *drosomycin* transgenes yielded proteins that purified with IMAC (Figure 2.17). Across three protein extractions and purifications the drosomycin fusion generally had better IMAC yields from the three elution's with highest protein concentration compared to the juruin fusion, 7.28 vs. 2.89 mg fusion per liter culture. Auto induction of by BL21 (DE3) is common and increased concentrations of IPTG did not show increased raw protein yield via Bradford assay or fusion protein yield via SDS-PAGE analysis for pLic-MBP-APETx2 derived vectors (data not shown).

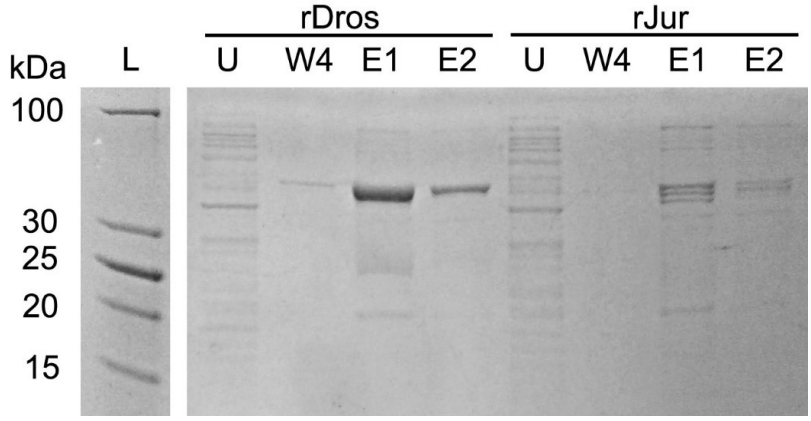


Figure 2.17 Affinity chromatography purification of recombinant AMP fusion proteins from *E. coli*

Steps in nickel column immobilized affinity chromatography purification of recombinant MBP drosomycin (rDros) and juruin (rJur) fusions from periplasm of *E. coli*. Lanes are unbound protein (U), wash four (W4), and elutions one (E1) and two (E2) against Page Ruler™ low range ladder (L). White strip in gel indicates gel splice. Predicted MW of rDros and rJur MBP fusions are 50,205 Da and 49,320 Da respectively.

For cleavage of fusion proteins with TEV protease reducing conditions allowed for much more efficient cleavage of the drosomycin fusion compared to the non-reducing (Figure 2.18). No discernible difference was seen between reducing and non-reducing conditions for the juruin

fusion. Following cleavage, recombinant proteins were separated from their fusion tag and TEVp by size exclusion filtration. The passthrough from these reactions did not show the presence of protein via Bradford assay. Fusion-free recombinant drosomycin was observed via SDS-PAGE silver staining (data not shown). In addition, MALDI-TOF confirmed the presence of a highly pure peptide in the mass range of drosomycin (Figure 2.19). Although there is evidence for a juruin fusion protein that is cleaved by TEVp, its presence was unconfirmed via SDS-PAGE or MALDI-TOF. This could be due to a low final concentration of the fusion free form. The sensitivity of MALDI-TOF could allow resolution of TEVp, fusion-tag, and POI if run directly on TEV reactions without a yield reducing size exclusion step.

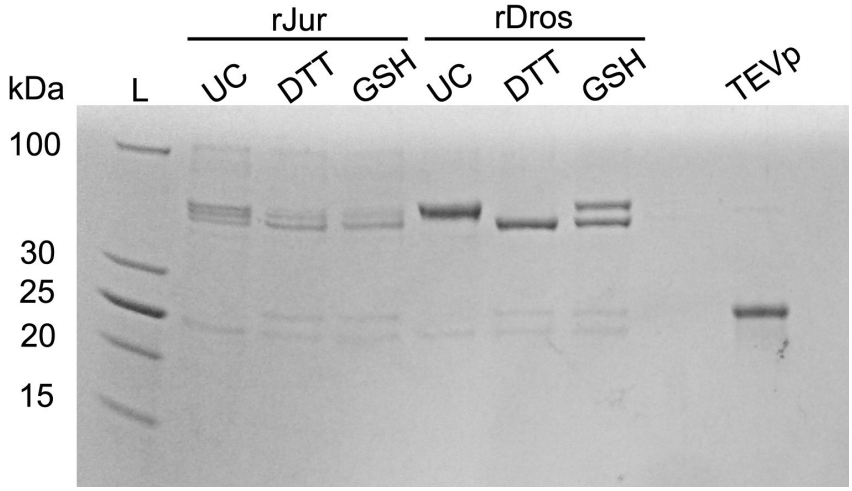


Figure 2.18 TEV cleavage of juruin and drosomycin MBP fusion proteins under different reaction conditions

Tobacco etch virus protease (TEVp) cleavage of juruin (rJur) and drosomycin (rDros) MBP fusions produced and purified from *E. coli.* TEV-free reactions, uncleaved (UC), compared to TEV cleavage under reducing (DTT) and non-reducing (GSH) conditions. Page Ruler™ low range ladder (L) was run for size estimation. In-house TEVp used in reactions was run alone as a control.

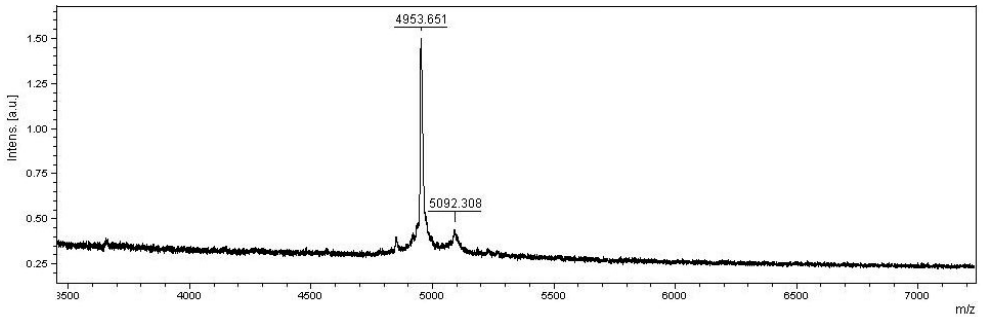


Figure 2.19 Mass spectrometry analysis of purified recombinant drosomycin protein derived from *E. coli*

MALDI-TOF MS spectrum of recombinant drosomycin produced from *E. coli* after TEV cleavage from MBP fusion and size exclusion with10 kDa cutoff filter. Predicted MW of recombinant drosomycin with glycine TEV cleavage scar is 4954.6 Da. Intensity in arbitrary units (Intens. [a.u.]) by mass-to-charge ratio (m/z).

Several things could be tried to increase the final yield of fusion-free AMP's in this procedure. Optimization of IMAC purification could be pursued by loading greater amounts of raw protein extract onto beads. Although cell disruption methods would undoubtedly increase raw protein yields, the premise of using periplasmic targeted fusion proteins such as used here is to have proteins fold correctly by the disulfide bond machinery present in the periplasm of *E. coli* (reviewed in: Berkmen 2012). Use of a strain of *E. coli* that is engineered to correctly fold disulfide bonded proteins in the cytoplasm could be a way around this (Lobstein, Emrich et al. 2012). Because TEV cleavage appears to be inefficient under non-reducing conditions, harvesting recombinant protein from the cytoplasm, where misfolding or unfolded protein is likely to occur, followed by TEV reactions under reducing conditions and subsequently using a protein refolding kit may be a strategy for producing large quantities of fusion protein. Also, re-designing plasmids to have multiple copies of a POI at one end of a fusion would increase the POI-to-fusion ratio and subsequent POI yield; however, this would leave additional TEV scar

residues at the termini of recombinant AMPs that could affect their activity. Other systems such as yeast and plants could also be used to produce these proteins.

Qualitative analysis of transgenic drosomycin and juruin wheat lines results and discussion

Since purification of recombinant juruin and drosomycin was unsuccessful at microgram quantities using *E. coli*, crude proteins were extracted from leaves of wheat expressing these transgenes. Presence or absence of the recombinant proteins could then be validated by several methods. First, presence of these proteins could be shown via greater inhibition of fungi *in vivo* compared to background or lines expressing other transgenes. Secondly, crude extracts could be analyzed by mass spectrometry to check for unique proteins corresponding to the size of juruin and drosomycin.

For a single *M. phaseolina* sclerotia type assay for isolates 65 and 110, no detectable inhibition was seen for LMPE from juruin 7079 T_3 and T_4, 7411 T_3, and 7398 T_2 lines while the nystatin positive control showed good inhibition (Figure 2.20). Results from experiments are shown in Table 2.2. Because of the small sample size, no significance could be determined between any of the treatments using Pearson's Chi-square test (smallest *p* value 0.10). Despite this, juruin lines showed better inhibition of both *N. crassa* and *M. phaseolina* compared to the negative control. A repeated experiment with the same results could show a significant difference between juruin line 7398 at 30 $\mu g\ mL^{-1}$ compared to the negative controls as well as Bobwhite treatments for *M. phaseolina* isolate 110. Increasing the concentration of LMPE may be required to see an inhibitory effect. LMPE's are composed not only of potential POI, but numerous other native proteins. This "undefined" extract dilutes the power of these types of

bioassays; however, the concentration of protein at the lowest concentration used here is one thousand times higher than that used to show inhibition against fungi for juruin (calculated from: Ayroza, Ferreira et al. 2012).

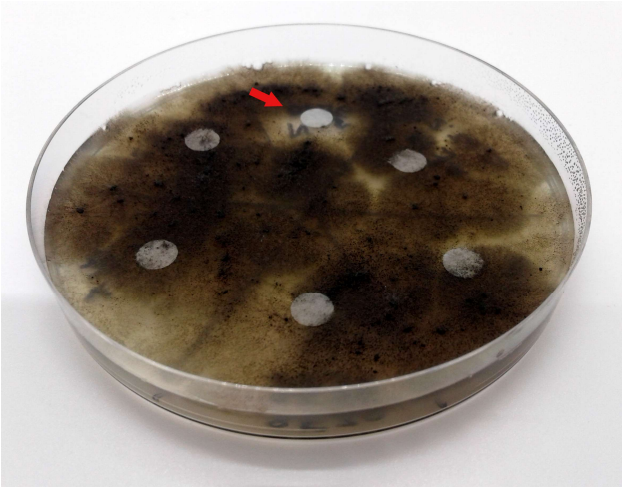


Figure 2.20 *Macrophomina phaseolina* sclerotia growth inhibition assay against wheat LMPE

Paper discs treated with 3-10 kDa wheat proteins from *juruin* and *drosomycin* expressing wheat lines. Red arrow indicates nystatin positive control and other discs are experimental treatments

N. crassa

	Pos Ctrl	Neg Ctrl	BW_{10}	BW_{30}	$6933A6_{10}$	$6933A6_{30}$	$7079A2_{10}$	$7079A2_{30}$	$7411B1_{10}$	$7411B1_{30}$	$7398A2_{10}$	$7398A_{30}$
inhibit	3	0	1	1	0	2	0	0	0	2	1	2
noninhibit	0	3	2	2	3	1	3	3	3	1	2	1

M.p. 65

	Pos Ctrl	Neg Ctrl	BW_{10}	BW_{30}	$6933A6_{10}$	$6933A6_{30}$	$7079A2_{10}$	$7079A2_{30}$	$7411B1_{10}$	$7411B1_{30}$	$7398A2_{10}$	$7398A_{30}$
inhibit	3	0	0	0	0	0	1	0	1	1	1	1
noninhibit	0	3	3	3	3	3	2	3	2	2	2	2

M.p. 110

	Pos Ctrl	Neg Ctrl	BW_{10}	BW_{30}	$6933A6_{10}$	$6933A6_{30}$	$7079A2_{10}$	$7079A2_{30}$	$7411B1_{10}$	$7411B1_{30}$	$7398A2_{10}$	$7398A_{30}$
inhibit	3	1	0	0	0	1	0	1	1	0	1	2
noninhibit	0	2	3	3	3	2	3	2	2	3	2	1

Table 2.2 *In vitro* assay results for *juruin* and *drosomycin* expressing wheat LMPE against several fungi

Inhibition/noninhibition of *Neurospora crassa* (*N. crassa*) and *Macrophomina phaseolina* (M.p.) isolates from nystatin (Pos Ctrl), 10% glycerol solution (Neg Ctrl), Bobwhite (BW), drosomycin (6933), and juruin (7079, 7411, and 7389). Concentration of experimental treatments in μg mL^{-1} are indicated in subscript.

The same LMPE from juruin line 7079 and drosomycin line 6933 used in the above bioassays were used for mass spec analysis. The first step of this process utilized RP-HPLC to separate proteins based on hydrophobicity. RP-HPLC of samples showed continuous 220 & 280 absorbance from 20-62 minutes. Experimental samples did not show unique peaks compared to the BW background for absorbance in either spectrum. Because proteins present in small amounts may be masked by the continuous absorbance produced by other proteins, the lack of a unique peak in HPLC analysis that would represent either recombinant juruin or drosomycin does not exclude their possible presence. Mass spec analysis showed distinguishable peaks throughout the 24-62 min fractions of elution for both juruin and drosomycin samples. An example of a fraction with peaks in the mass range of juruin can be seen in Figure 2.21.A. No unique peaks were observed for juruin fractions when compared to drosomycin fractions for mass spec analysis. No unique peaks were found for drosomycin near its predicted MW of 4.89 kDa. Highest discernible peaks for drosomycin fractions for mass spec analysis was 6.59 kDa. This excluded the possibility of observing its potential preprotein at 7.75 kDa. Although

expression of proteins from pAHC17 with its *Ubi-1* exon and intron have been reported to yield the correct size recombinant proteins, there is still the possibility of alternative splicing (Toki, Takamatsu et al. 1992). When produced in yeast, drosomycin with its typical mass of 4.89 kDa accounts for only 25% of the recombinant drosomycin recovered. The remaining is drosomycin as one of three alternatively processed forms with extra N-terminal residues giving it a MW of up to 6.37 kDa (Michaut, Fehlbaum et al. 1996). Presence of unique peaks such as those seen in the electronic gel in Figure 2.21.B could possibly be alternatively processed drosomycin. Absence of unique peaks does not eliminate the possibility of protein product from transgenes. As with HPLC analysis, native protein peaks at the same MW as predicted recombinant AMPs could mask the presence of juruin and drosomycin. Isolation of peaks and performing N-terminal sequencing may reveal their presence. Modifications of the recombinant proteins, such as glycosylation or alternate processing, by their new host can make identifying their presence problematic. Mass spec analysis needs to be run on more lines to check for the presence of recombinant juruin and drosomycin in wheat lines expressing their transgenes. Increased initial LMPE may be essential for determining their presence through mass spec and inhibition of fungi *in vitro* as it is common for recombinant proteins harvested from plants to make a fraction of a percent to total soluble protein (Kusnadi, Nikolov et al. 1997).

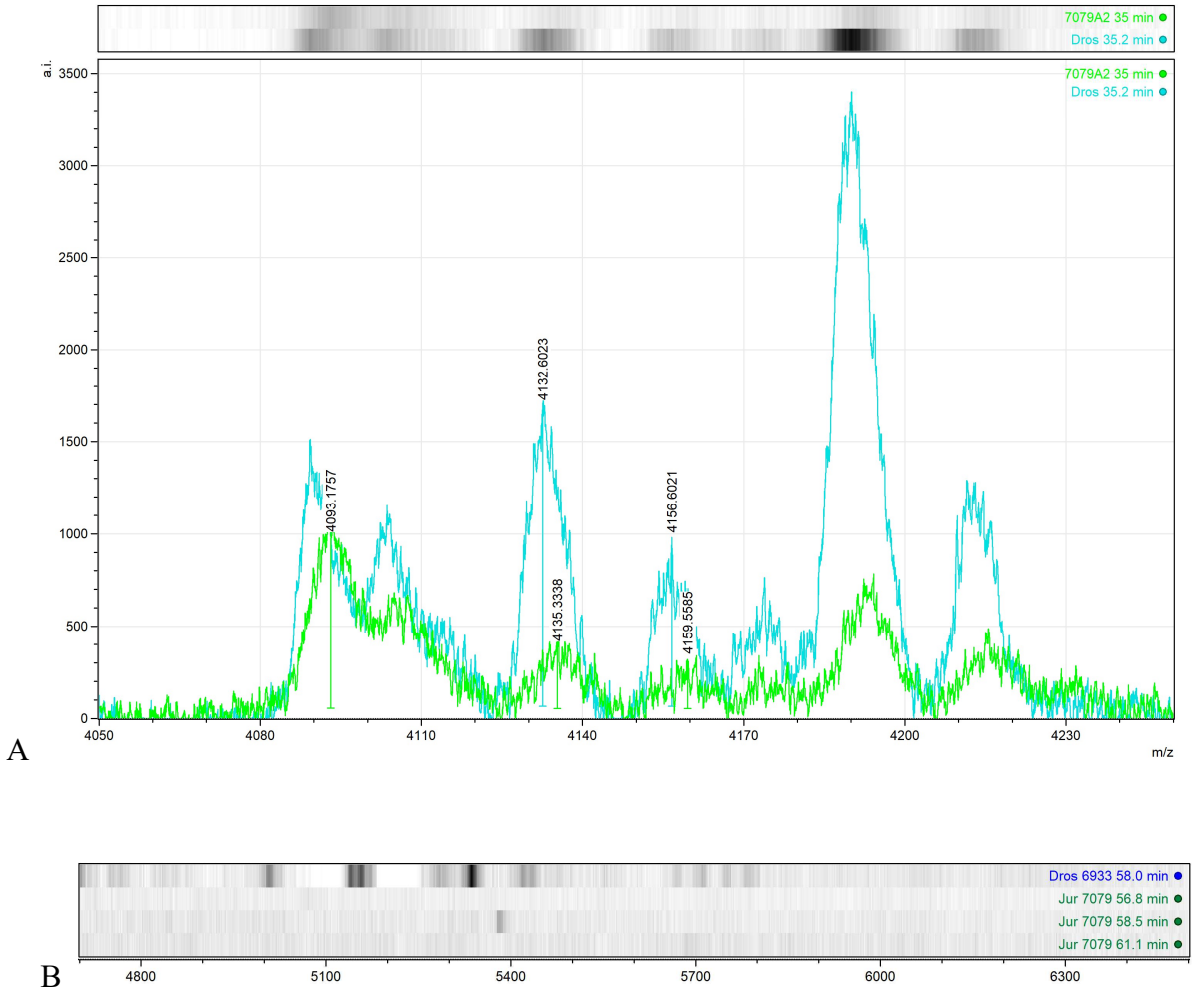


Figure 2.21 Mass spec analysis of LMPE's from wheat

MALDI-TOF spectra for LMPE from wheat expressing *juruin* (7079A2, light green) compared to *drosomycin* expressing wheat (6933 light blue) from RP-HPLC fractions ~35 min (A). Electronic gel showing unique peaks from drosomycin line compared to same juruin factions (B). Arbitrary absorbance (a.i.) against mass-to-charge ratio (m/z). Peaks of interest labeled by mass.

Works Cited

(2010). Compendium of wheat diseases and pests. St. Paul, MN, American Phytopathological Society.

Adams, J. R., M. E. Bailey, C. E. Burkhead, H. T. Cook, J. Vermeer, R. C. Wilson and E. L. LeClerg (1965). Losses in agriculture. U. S. D. o. Agriculture.

Altschul, S. F., T. L. Madden, A. A. Schäffer, J. Zhang, Z. Zhang, W. Miller and D. J. Lipman (1997). "Gapped blast and psi-blast: A new generation of protein database search programs." Nucleic Acids Research **25**(17): 3389-3402.

Anagnostakis, S. L. (1987). "Chestnut blight: The classical problem of an introduced pathogen." Mycologica **79**: 23-37.

Anangi, R., L. D. Rash, M. Mobli and G. F. King (2012). "Functional expression in *escherichia coli* of the disulfide-rich sea anemone peptide apetx2, a potent blocker of acid-sensing ion channel 3." Mar Drugs **10**(7): 1605-1618.

Anderson, T. R., H. Olechowski and T. Welacky (1988). "Incidence of rhizoctonia and fusarium root rot of soybean in southwestern ontario, 1986." Canadian Plant Disease Survey **68**: 143-145.

Annone, J. G. (1997). Tan spot of wheat in argentina: Importance and disease managment strategies. Helminthosporium blights of wheat: Spot blotch and tan spot**:** 339-345.

Appel, J. A., E. DeWolf, T. Todd and W. W. Bockus (2015). Preliminary 2015 kansas wheat disease loss estimates. Kansas Cooperative Plant Disease Survey Report.

Atwood, D. and C. Paisley-Jones (2017). Pesticides industry sales and usage: 2008-2012 market estimates. U. S. E. P. Agency.

Ayroza, G., I. L. Ferreira, R. S. Sayegh, A. K. Tashima and P. I. da Silva Junior (2012). "Juruin: An antifungal peptide from the venom of the amazonian pink toe spider, avicularia juruensis, which contains the inhibitory cystine knot motif." Front Microbiol **3**: 324.

Bai, G., Z. Su and J. Cai (2018). "Wheat resistance to fusarium head blight." Canadian Journal of Plant Pathology **40**(3): 336-346.

Ballance, G. M., L. Lamari and C. C. Bernier (1989). "Purification and characterization of a host-selective necrosis toxin from *pyrenophora tritici-repentis*." Physiological and Molecular Plant Pathology **35**(3): 203-213.

Banno, S., F. Fukumori, A. Ichiishi, K. Okada, H. Uekusa, M. Kimura and M. Fujimura (2008). "Genotyping of benzimidazole-resistant and dicarboximide-resistant mutations in botrytis cinerea using real-time polymerase chain reaction assays." Phytopathology **98**(4): 397-404.

Batalia, M. A., A. F. Monzingo, S. Ernst, W. Roberts and J. D. Robertus (1996). "The crystal structure of the antifungal protein zeamatin, a member of the thaumatin-like, pr-5 protein family." Nat Struct Biol **3**(1): 19-23.

Berkmen, M. (2012). "Production of disulfide-bonded proteins in escherichia coli." Protein Expression and Purification **82**(1): 240-251.

Bi, Y. M., B. P. A. Cammue, P. H. Goodwin, S. KrishnaRaj and P. K. Saxena (1999). "Resistance to botrytis cinerea in scented geranium transformed with a gene encoding the antimicrobial protein ace-amp1." Plant Cell Reports **18**(10): 835-840.

Bjorksten, F., L. Halmepuro, M. Hannuksela and A. Lahti (1980). "Extraction and properties of apple allergens." Allergy **35**(8): 671-677.

Bockus, W. W. and M. M. Claassen (1992). "Effects of crop rotation and residue managment practices on severity of tan spot of winter wheat." Plant Disease **76**: 633-636.

Bruns, H. A. (2017). "Sourthern corn leaf blight: A story worth retelling." **109**(4): 1-7.

Burgess, L., A. Wearing and T. Toussoun (1975). "Surveys of fusaria associated with crown rot of wheat in eastern australia." Australian Journal of Agricultural Research **26**(5): 791-799.

Bushnell, W. R., B. E. Hazen and C. Pritsch (2003). Histology and physiology of fusarium head blight. Fusarium head blight of wheat and barley. K. J. Leonard and W. R. Bushnell**:** 44-83.

Cammue, B. P. A., K. Thevissen, M. Hendriks, K. Eggermont, I. J. Goderis, P. Proost, J. Van Damme, R. W. Osborn, F. Guerbette, J. C. Kader and W. F. Broekaert (1995). "A potent antimicrobial protein from onion seeds showing sequence homology to plant lipid transfer proteins." Plant Physiology **109**(2): 445.

Cheng, W., X.-S. Song, H.-P. Li, L.-H. Cao, K. Sun, X.-L. Qiu, Y.-B. Xu, P. Yang, T. Huang, J.-B. Zhang, B. Qu and Y.-C. Liao (2015). "Host-induced gene silencing of an essential chitin synthase gene confers durable resistance to fusarium head blight and seedling blight in wheat." Plant Biotechnology Journal **13**(9): 1335-1345.

Chongo, G., B. D. Gossen, H. R. Kutcher, J. Gilbert, T. K. Turkington, M. R. Fernandez and D. McLaren (2001). "Reaction of seedling roots of 14 crop species to *fusarium graminearum* from wheat heads." Canadian Journal of Plant Pathology **23**(2): 132-137.

Christensen, A. H. and P. H. Quail (1996). "Ubiquitin promoter-based vectors for high-level expression of selectable and/or screenable marker genes in monocotyledonous plants." Transgenic Res **5**(3): 213-218.

Christensen, J. J., E. C. Stakman and F. R. Immer (1929). Susceptibility of wheat varieties and hybrids to fusarial head blight in minnesota, University of Minnesota, Minnesota Agricultural Experiment Station.

Chu, H., M. Pazgier, G. Jung, S. P. Nuccio, P. A. Castillo, M. F. de Jong, M. G. Winter, S. E. Winter, J. Wehkamp, B. Shen, N. H. Salzman, M. A. Underwood, R. M. Tsolis, G. M. Young, W. Lu, R. I. Lehrer, A. J. Baumler and C. L. Bevins (2012). "Human alpha-defensin 6 promotes mucosal innate immunity through self-assembled peptide nanonets." Science **337**(6093): 477-481.

de Beer, A. and M. A. Vivier (2011). "Four plant defensins from an indigenous south african brassicaceae species display divergent activities against two test pathogens despite high sequence similarity in the encoding genes." BMC Res Notes **4**: 459.

De Lucca, A. J. and T. J. Walsh (1999). "Antifungal peptides: Novel therapeutic compounds against emerging pathogens." Antimicrob Agents Chemother **43**(1): 1-11.

De Wolf, E. D., R. J. Effertz, S. Ali and L. J. Francl (1998). "Vistas of tan spot research." Canadian Journal of Plant Pathology **20**(4): 349-370.

Dickson, J. G., H. Johann and G. Wineland (1921). "Second progress report on the fusarium blight (scab) of wheat." Phytopathology **11**.

Dimitrov, I., D. R. Flower and I. Doytchinova (2013). "Allertop--a server for in silico prediction of allergens." BMC bioinformatics **14 Suppl 6**(Suppl 6): S4-S4.

Duran, R. and G. W. Fisher (1961). The genus tilletia. Pullman, WA, Washington State University Press.

Effertz, R. J., S. W. Meinhardt, J. A. Anderson, J. G. Jordahl and L. J. Francl (2002). "Identification of a chlorosis-inducing toxin from *pyrenophora tritici-repentis* and the chromosomal location of an insensitivity locus in wheat." Phytopathology **92**(5): 527-533.

El-Mounadi, K., K. T. Islam, P. Hernandez-Ortiz, N. D. Read and D. M. Shah (2016). "Antifungal mechanisms of a plant defensin mtdef4 are not conserved between the ascomycete fungi *neurospora crassa* and *fusarium graminearum*." Mol Microbiol **100**(3): 542-559.

Escoubas, P. and L. Rash (2004). "Tarantulas: Eight-legged pharmacists and combinatorial chemists." Toxicon **43**(5): 555-574.

Fehlbaum, P., P. Bulet, L. Michaut, M. Lagueux, W. F. Broekaert, C. Hetru and J. A. Hoffmann (1994). "Insect immunity. Septic injury of drosophila induces the synthesis of a potent antifungal peptide with sequence homology to plant antifungal peptides." J Biol Chem **269**(52): 33159-33163.

Fernández-Ortuño, D., A. Pérez-García, F. López-Ruiz, D. Romero, A. de Vicente and J. A. Torés (2006). "Occurrence and distribution of resistance to qoi fungicides in populationsof podosphaera fusca in south central spain." European Journal of Plant Pathology **115**(2): 215-222.

Fernandez de Caleya, R., B. Gonzalez-Pascual, F. Garcia-Olmedo and P. Carbonero (1972). "Susceptibility of phytopathogenic bacteria to wheat purothionins in vitro." Appl Microbiol **23**(5): 998-1000.

Fernandez, M. R., R. M. DePauw and J. M. Clarke (2001). "Reaction of common and durum wheat cultivars to infection of kernels by *pyrenophora tritici-repentis*." Canadian Journal of Plant Pathology **23**(2): 158-162.

Fernandez, M. R. and J. M. C. Fernandas (1990). "Survival of wheat pathogens in wheat and soybean residues under conservation tillage systems in southern and central brazil." Canadian Journal of Plant Pathology **12**(3): 289-294.

Fischer, G. W. and C. S. Holton (1957). Life history and parasitism. Biology and control of the smut fungi, Ronald Press Company**:** 144-220.

Fletcher, S. J. (2014). "Qpcr for quantification of transgene expression and determination of transgene copy number." Methods Mol Biol **1145**: 213-237.

Fofana, B., D. G. Humphreys, S. Cloutier, C. A. McCartney and D. J. Somers (2008). "Mapping quantitative trait loci controlling common bunt resistance in a doubled haploid population derived from the spring wheat cross rl4452 × ac domain." Molecular Breeding **21**(3): 317-325.

Gaudet, D. A. and A. Laroche (1998). "Breeding for resistance to common bunt: A canadian perspective." Bunts and Smuts of Wheat: An International Symposium: 363.

Goates, B. J. (2011). "Identification of new pathogenic races of common bunt and dwarf bunt fungi, and evaluation of known races using an expanded set of differential wheat lines." Plant Disease **96**(3): 361-369.

Goyal, R. K. and A. K. Mattoo (2014). "Multitasking antimicrobial peptides in plant development and host defense against biotic/abiotic stress." Plant Science **228**: 135-149.

Graham, J. S., W. Burkhart, J. Xiong and J. W. Gillikin (1992). "Complete amino acid sequence of soybean leaf p21 : Similarity to the thaumatin-like polypeptides." Plant Physiol **98**(1): 163-165.

Gratia, A. (1925). "Sur un remarquable exemple d'antagonisme entre deux souches de colibacille." Comptes Rendus des Séances et Mémoires de la Société de Biologie **93**: 1040-1041.

Guo, B. Z., Z. Y. Chen, R. L. Brown, A. R. Lax, T. E. Cleveland, J. S. Russin, A. D. Mehta, C. P. Selitrennikoff and N. W. Widstrom (1997). "Germination induces accumulation of specific proteins and antifungal activities in corn kernels." Phytopathology **87**(11): 1174-1178.

Haney, E. F., S. C. Mansour and R. E. W. Hancock (2017). Antimicrobial peptides: An introduction. Antimicrobial peptides: Methods and protocols. P. R. Hansen. New York, NY, Springer New York**:** 3-22.

Haskell, R. J., R. W. Leukel and E. G. Boerner (1931). Stinking smut (bunt) in wheat and how to prevent it. U. S. D. o. Agriculture. Washington, D. C.

Hernandez-Lucas, C., R. Fernandez de Caleya and P. Carbonero (1974). "Inhibition of brewer's yeasts by wheat purothionins." Applied microbiology **28**(2): 165-168.

Hoffmann, J. A. and J. T. Waldher (1981). "Chemical seed treatments for controlling seedborne and soilborne common bunt of wheat." Plant Disease **65**: 256-259.

Ingolia, N. T., S. Ghaemmaghami, J. R. S. Newman and J. S. Weissman (2009). "Genome-wide analysis in vivo of translation with nucleotide resolution using ribosome profiling." Science (New York, N.Y.) **324**(5924): 218-223.

Islam, M. T., D. Croll, P. Gladieux, D. M. Soanes, A. Persoons, P. Bhattacharjee, M. S. Hossain, D. R. Gupta, M. M. Rahman, M. G. Mahboob, N. Cook, M. U. Salam, M. Z. Surovy, V. B. Sancho, J. L. N. Maciel, A. NhaniJúnior, V. L. Castroagudín, J. T. d. A. Reges, P. C. Ceresini, S. Ravel, R. Kellner, E. Fournier, D. Tharreau, M.-H. Lebrun, B. A. McDonald, T. Stitt, D. Swan, N. J. Talbot, D. G. O. Saunders, J. Win and S. Kamoun (2016). "Emergence of wheat blast in bangladesh was caused by a south american lineage of magnaporthe oryzae." BMC Biology **14**(1): 84.

Jang, W. S., S.-C. Lee, Y. S. Lee, Y. P. Shin, K. H. Shin, B. H. Sung, B. S. Kim, S. H. Lee and I. H. Lee (2007). "Antimicrobial effect of halocidin-derived peptide in a mouse model of *listeria*; infection." Antimicrobial Agents and Chemotherapy **51**(11): 4148.

Jardine, D. (2014). "Soybean disease managment update." 2019, from https://www.agronomy.k-state.edu/extension/documents/soybean-schools/2014-soybean-school/Jardine-Soybean-2014.pdf.

Johnsson, L. (1990). "Survival of common bunt [tilletia caries (dc) tul.) in soil and manure / das überleben von sporen des gewöhnlichen steinbrands (tilletia caries (dc) tul.) in erde und dünger." Zeitschrift für Pflanzenkrankheiten und Pflanzenschutz / Journal of Plant Diseases and Protection **97**(5): 502-507.

Kanzaki, H., S. Nirasawa, H. Saitoh, M. Ito, M. Nishihara, R. Terauchi and I. Nakamura (2002). "Overexpression of the wasabi defensin gene confers enhanced resistance to blast fungus (magnaporthe grisea) in transgenic rice." Theor Appl Genet **105**(6-7): 809-814.

Kim, D., R. Jeannotte, R. Welti and W. W. Bockus (2012). "Lipid profiles in wheat cultivars resistant and susceptible to tan spot and the effect of disease on the profiles." Phytopathology **103**(1): 74-80.

Klepikova, A. V., A. S. Kasianov, E. S. Gerasimov, M. D. Logacheva and A. A. Penin (2016). "A high resolution map of the arabidopsis thaliana developmental transcriptome based on rna-seq profiling." The Plant Journal **88**(6): 1058-1070.

Krishnamurthy, K., C. Balconi, J. E. Sherwood and M. J. Giroux (2001). "Wheat puroindolines enhance fungal disease resistance in transgenic rice." Molecular Plant-Microbe Interactions® **14**(10): 1255-1260.

Kusnadi, A. R., Z. L. Nikolov and J. A. Howard (1997). "Production of recombinant proteins in transgenic plants: Practical considerations." Biotechnology and Bioengineering **56**(5): 473-484.

Lai, R., H. Liu, W. Hui Lee and Y. Zhang (2002). "An anionic antimicrobial peptide from toad bombina maxima." Biochem Biophys Res Commun **295**(4): 796-799.

Lamari, L., R. Sayoud, M. Boulif and C. C. Bernier (1995). "Identification of a new race in *pyrenophora tritici-repentis*: Implications for the current pathotype classification system." Canadian Journal of Plant Pathology **17**(4): 312-318.

Landon, C., P. Sodano, C. Hetru, J. Hoffmann and M. Ptak (1997). "Solution structure of drosomycin, the first inducible antifungal protein from insects." Protein Sci **6**(9): 1878-1884.

Li, X., K. Gasic, B. Cammue, W. Broekaert and S. S. Korban (2003). "Transgenic rose lines harboring an antimicrobial protein gene, ace-amp1, demonstrate enhanced resistance to powdery mildew (sphaerotheca pannosa)." Planta **218**(2): 226-232.

Li, Z., M. Zhou, Z. Zhang, L. Ren, L. Du, B. Zhang, H. Xu and Z. Xin (2011). "Expression of a radish defensin in transgenic wheat confers increased resistance to *fusarium graminearum* and *rhizoctonia cerealis*." Functional & Integrative Genomics **11**(1): 63-70.

Liu, J., S. Zhang and L. Li (2009). "A transferrin-like homolog in amphioxus branchiostoma belcheri: Identification, expression and functional characterization." Mol Immunol **46**(15): 3117-3124.

Liu, J. J., R. Sturrock and A. K. Ekramoddoullah (2010). "The superfamily of thaumatin-like proteins: Its origin, evolution, and expression towards biological function." Plant Cell Rep **29**(5): 419-436.

Liu, S., M. D. Hall, C. A. Griffey and A. L. McKendry (2009). "Meta-analysis of qtl associated with fusarium head blight resistance in wheat." Crop Science **49**(6): 1955-1968.

Lobstein, J., C. A. Emrich, C. Jeans, M. Faulkner, P. Riggs and M. Berkmen (2012). "Shuffle, a novel escherichia coli protein expression strain capable of correctly folding disulfide bonded proteins in its cytoplasm." Microb Cell Fact **11**: 56.

Lu, Z. X., D. A. Gaudet, M. Frick, B. Puchalski, B. Genswein and A. Laroche (2005). "Identification and characterization of genes differentially expressed in the resistance reaction in wheat infected with tilletia tritici, the common bunt pathogen." J Biochem Mol Biol **38**(4): 420-431.

Mardirossian, M., R. Grzela, C. Giglione, T. Meinnel, R. Gennaro, P. Mergaert and M. Scocchi (2014). "The host antimicrobial peptide bac71-35 binds to bacterial ribosomal proteins and inhibits protein synthesis." Chemistry & Biology **21**(12): 1639-1647.

Markell, S. G. and L. J. Francl (2003). "Fusarium head blight inoculum: Species prevalence and *gibberella zeae* spore type." Plant Disease **87**(7): 814-820.

McCarthy, P. J., P. F. Troke and K. Gull (1985). "Mechanism of action of nikkomycin and the peptide transport system of candida albicans." J Gen Microbiol **131**(4): 775-780.

McMullen, M., R. Jones and D. Gallenberg (1997). "Scab of wheat and barley: A re-emerging disease of devastating impact." Plant Disease **81**(12): 1340-1348.

Michaut, L., P. Fehlbaum, M. Moniatte, A. Van Dorsselaer, J. M. Reichhart and P. Bulet (1996). "Determination of the disulfide array of the first inducible antifungal peptide from insects: Drosomycin from drosophila melanogaster." FEBS Lett **395**(1): 6-10.

Neu, H. C. and L. A. Heppel (1965). "The release of enzymes from *escherichia coli* by osmotic shock and during the formation of spheroplasts." J Biol Chem **240**(9): 3685-3692.

Neukermans, J., A. Inze, J. Mathys, B. De Coninck, B. van de Cotte, B. P. Cammue and F. Van Breusegem (2015). "Aracins, brassicaceae-specific peptides exhibiting antifungal activities against necrotrophic pathogens in arabidopsis." Plant Physiol **167**(3): 1017-1029.

Neumann, G. M., R. Condron, I. Thomas and G. M. Polya (1994). "Purification and sequencing of a family of wheat lipid transfer protein homologues phosphorylated by plant calcium-dependent protein kinase." Biochim Biophys Acta **1209**(2): 183-190.

Newhouse, A. E., L. D. Polin-McGuigan, K. A. Baier, K. E. Valletta, W. H. Rottmann, T. J. Tschaplinski, C. A. Maynard and W. A. Powell (2014). "Transgenic american chestnuts show enhanced blight resistance and transmit the trait to t1 progeny." Plant Sci **228**: 88-97.

Nicolas, P. (2009). "Multifunctional host defense peptides: Intracellular-targeting antimicrobial peptides." FEBS J **276**(22): 6483-6496.

Park, C. J., C. B. Park, S. S. Hong, H. S. Lee, S. Y. Lee and S. C. Kim (2000). "Characterization and cdna cloning of two glycine- and histidine-rich antimicrobial peptides from the roots of shepherd's purse, capsella bursa-pastoris." Plant molecular biology **44**(2): 187-197.

Patiño, B., C. Vázquez, J. M. Manning, M. I. G. Roncero, D. Córdoba-Cañero, A. Di Pietro and Á. Martínez-del-Pozo (2018). "Characterization of a novel cysteine-rich antifungal protein from *fusarium graminearum* with activity against maize fungal pathogens." International Journal of Food Microbiology **283**: 45-51.

Patkar, R. N. and B. B. Chattoo (2006). "Transgenic indica rice expressing ns-ltp-like protein shows enhanced resistance to both fungal and bacterial pathogens." Molecular Breeding **17**(2): 159-171.

Pawlowski, W. P. and D. A. Somers (1996). "Transgene inheritance in plants genetically engineered by microprojectile bombardment." Mol Biotechnol **6**(1): 17-30.

Pérez, P., V. Rosa, G.-A. Isabel and D. Angel (1981). "Effect of papulacandin b and aculeacin a on β-(1,3) glucan-synthase from geotrichum lactis." FEBS Letters **129**(2): 249-252.

Pirtskhalava, M., A. Gabrielian, P. Cruz, H. L. Griggs, R. B. Squires, D. E. Hurt, M. Grigolava, M. Chubinidze, G. Gogoladze, B. Vishnepolsky, V. Alekseyev, A. Rosenthal and M. Tartakovsky (2016). "Dbaasp v.2: An enhanced database of structure and antimicrobial/cytotoxic activity of natural and synthetic peptides." Nucleic Acids Res **44**(13): 6503.

Proctor, R. H., T. M. Hohn and S. P. McCormick (1995). "Reduced virulence of gibberella zeae caused by disruption of a trichothecene toxin biosynthetic gene." Mol Plant Microbe Interact **8**(4): 593-601.

Rasmussen-Wilson, S. J., J. S. Palas, V. J. Wolf, C. S. Taft and C. P. Selitrennikoff (1997). "Expression of a plant protein by neurospora crassa." Appl Environ Microbiol **63**(9): 3488-3493.

Rees, R. G. and G. J. Platz (1983). "Effects of yellow spot on wheat: Comparison of epidemics at different stages of crop development." Australian Journal of Agricultural Research **34**(1): 39-46.

Rigling, D. and S. Prospero (2018). "Cryphonectria parasitica, the causal agent of chestnut blight: Invasion history, population biology and disease control." Mol Plant Pathol **19**(1): 7-20.

Robert, V., G. Stegehuis and J. Stalpers. (2005). "The mycobank engine and related databases." Retrieved 3/30/2020, 2020, from http://www.mycobank.org.

Roberts, W. K. and C. P. Selitrennikoff (1990). "Zeamatin, an antifungal protein from maize with membrane-permeabilizing activity." Journal of General Microbiology **136**: 1771-1778.

Russell, B. W. and D. Mills (1994). "Morphological, physiological, and genetic evidence in support of a conspecific status for tilletia caries, t. Controversa, and t. Foetida." The American Phytopathological Society **84**: 576-582.

Saitoh, H., A. Kiba, M. Nishihara, S. Yamamura, K. Suzuki and R. Terauchi (2001). "Production of antimicrobial defensin in nicotiana benthamiana with a potato virus x vector." Molecular Plant-Microbe Interactions® **14**(2): 111-115.

Sasaki, K., C. Kuwabara, N. Umeki, M. Fujioka, W. Saburi, H. Matsui, F. Abe and R. Imai (2016). "The cold-induced defensin tad1 confers resistance against snow mold and fusarium head blight in transgenic wheat." Journal of Biotechnology **228**: 3-7.

Scott, I. T. (1927). Varietal resistance and susceptibility to wheat scab, University of Missouri.

Shabeer, A. and W. W. Bockus (1988). "Tan spot effects on yield and yield components relative to growth stage in winter wheat." Plant Disease **72**(7): 599-602.

Sharma, P. and A. Lonneborg (1996). "Isolation and characterization of a cdna encoding a plant defensin-like protein from roots of norway spruce." Plant Mol Biol **31**(3): 707-712.

Shi, Y. L., P. Loomis, D. Christian, L. M. Carris and H. Leung (1996). "Analysis of the genetic relationships among the wheat bunt fungi using rapd and ribosomal DNA markers." Phytopathology **86**: 311-318.

Silverstein, K. A. T., M. A. Graham, T. D. Paape and K. A. VandenBosch (2005). "Genome organization of more than 300 defensin-like genes in arabidopsis." Plant Physiology **138**(2): 600.

Sinha, R. and P. Shukla (2019). "Antimicrobial peptides: Recent insights on biotechnological interventions and future perspectives." Protein and peptide letters **26**(2): 79-87.

Stack, R. W. (1989). "A comparison of the inoculum potential of ascospores and conidia of gibberella zeae." Canadian Journal of Plant Pathology **11**(2): 137-142.

Stack, R. W. (2003). History of fusarium head blight with emphasis on north america. Fusarium head blight of wheat and barley. K. J. Leonard and W. R. Bushnell, American Phytopathological Society**:** 1-34.

Stevens, D. A., L. Calderon, M. Martinez, K. V. Clemons, S. J. Wilson and C. P. Selitrennikoff (2002). "Zeamatin, clotrimazole and nikkomycin z in therapy of a candida vaginitis model." J Antimicrob Chemother **50**(3): 361-364.

Strange, R. N. and H. Smith (1978). "Specificity of choline and betaine as stimulants of *fusarium graminearum*." Transactions of the British Mycological Society **70**(2): 187-192.

Strelkov, S. E., L. Lamari and G. M. Ballance (1999). "Characterization of a host-specific protein toxin (ptr toxb) from *pyrenophora tritici-repentis*." Molecular Plant-Microbe Interactions® **12**(8): 728-732.

Sunisha, C., H. D. Sowmya, T. R. Usharani, M. Umesha, H. R. Gopalkrishna and A. Saxena (2019). "Deployment of stacked antimicrobial genes in banana for stable tolerance against *fusarium oxysporum* f.Sp. *Cubense* through genetic transformation." Molecular Biotechnology.

Sutton, J. C. (1982). "Epidemiology of wheat head blight and maize ear rot caused by fusarium graminearum." Canadian Journal of Plant Pathology **4**(2): 195-209.

Swinburne, T. R. (1963). "Infection of wheat by tilletia caries (dc.) tul., the causal organism of bunt." Transactions of the British Mycological Society **46**(1): 145-IN112.

Tam, J. P., S. Wang, K. H. Wong and W. L. Tan (2015). "Antimicrobial peptides from plants." Pharmaceuticals (Basel, Switzerland) **8**(4): 711-757.

Tassin, S., W. F. Broekaert, D. Marion, D. P. Acland, M. Ptak, F. Vovelle and P. Sodano (1998). "Solution structure of ace-amp1, a potent antimicrobial protein extracted from onion

seeds. Structural analogies with plant nonspecific lipid transfer proteins." Biochemistry **37**(11): 3623-3637.

Tian, B., M. Navia-Urrutia, Y. Chen, J. Brungardt and H. N. Trick (2019). "Biolistic transformation of wheat." Methods Mol Biol **1864**: 117-130.

Tillet, M. (1755). "Dissertation on the cause of the corruption and smutting of the kernels of wheat in the head and on the means of preventing these untoward circumstances."

Toki, S., S. Takamatsu, C. Nojiri, S. Ooba, H. Anzai, M. Iwata, A. H. Christensen, P. H. Quail and H. Uchimiya (1992). "Expression of a maize ubiquitin gene promoter-bar chimeric gene in transgenic rice plants." Plant physiology **100**(3): 1503-1507.

Tu, C. (1930). Physiological specialization in *fusarium* spp. Causing headblight of small grains., Minnesota Agricultural Experiment Station Technical Bulletin**:** 27.

USDA, N. A. S. S. National satistics for wheat; price recieved in $/bu, USDA-NASS.

van der Lee, T., H. Zhang, A. van Diepeningen and C. Waalwijk (2015). "Biogeography of *fusarium graminearum* species complex and chemotypes: A review." Food additives & contaminants. Part A, Chemistry, analysis, control, exposure & risk assessment **32**(4): 453-460.

Vandesompele, J., K. De Preter, F. Pattyn, B. Poppe, N. Van Roy, A. De Paepe and F. Speleman (2002). "Accurate normalization of real-time quantitative rt-pcr data by geometric averaging of multiple internal control genes." Genome Biology **3**(7): research0034.0031.

Vasconcelos, I. M., J. K. Morais, E. A. Siebra, C. R. Carlini, D. O. Sousa, L. M. Beltramini, V. M. Melo and J. T. Oliveira (2008). "Sbtx, a new toxic protein distinct from soyatoxin and other toxic soybean [*glycine max*] proteins, and its inhibitory effect on *cercospora sojina* growth." Toxicon **51**(6): 952-963.

Waghu, F. H., R. S. Barai, P. Gurung and S. Idicula-Thomas (2016). "Campr3: A database on sequences, structures and signatures of antimicrobial peptides." Nucleic Acids Res **44**(D1): D1094-1097.

Wang, G., X. Li and Z. Wang (2008). "Apd2: The updated antimicrobial peptide database and its application in peptide design." Nucleic Acids Research **37**(suppl_1): D933-D937.

Wang, X., C. Tang, L. Deng, G. Cai, X. Liu, B. Liu, Q. Han, H. Buchenauer, G. Wei, D. Han, L. Huang and Z. Kang (2010). "Characterization of a pathogenesis-related thaumatin-like protein gene tapr5 from wheat induced by stripe rust fungus." Physiol Plant **139**(1): 27-38.

Wilcoxson, R. D. and E. E. Saari, Eds. (1996). Bunt and smut diseases of wheat: Concepts and methods of disease managment. Mexico, D. F., CIMMYT.

Wise, K. (2016). Fungicide efficacy for control of wheat diseases, Purdue Extension.

Wong, J. H. and T. B. Ng (2006). "Limenin, a defensin-like peptide with multiple exploitable activities from shelf beans." Journal of Peptide Science **12**(5): 341-346.

Wu, Y., Y. He and X. Ge (2011). "Functional characterization of the recombinant antimicrobial peptide trx-ace-amp1 and its application on the control of tomato early blight disease." Applied Microbiology and Biotechnology **90**(4): 1303-1310.

Yadav, D., Y. Shavrukov, N. Bazanova, L. Chirkova, N. Borisjuk, N. Kovalchuk, A. Ismagul, B. Parent, P. Langridge, M. Hrmova and S. Lopato (2015). "Constitutive overexpression of the tanf-yb4 gene in transgenic wheat significantly improves grain yield." Journal of experimental botany **66**(21): 6635-6650.

Yang, L.-N., M.-H. He, H.-B. Ouyang, W. Zhu, Z.-C. Pan, Q.-J. Sui, L.-P. Shang and J. Zhan (2019). "Cross-resistance of the pathogenic fungus alternaria alternata to fungicides with different modes of action." BMC Microbiology **19**(1): 205.

Yang, W.-Y., S.-Y. Wen, Y.-D. Huang, M.-Q. Ye, X.-J. Deng, D. Han, Q.-Y. Xia and Y. Cao (2006). "Functional divergence of six isoforms of antifungal peptide drosomycin in drosophila melanogaster." Gene **379**: 26-32.

Yu, G., X. Zhang, J. Yao, M. Zhou and H. Ma (2017). "Resistance against fusarium head blight in transgenic wheat plants expressing the scnpr1 gene." Journal of Phytopathology **165**(4): 223-231.

Yuan, Y., B. Gao and S. Zhu (2007). "Functional expression of a drosophila antifungal peptide in escherichia coli." Protein Expr Purif **52**(2): 457-462.

Zhang, Z. and S. Zhu (2010). "Functional role of charged residues in drosomycin, a drosophila antifungal peptide." Developmental & Comparative Immunology **34**(9): 953-958.

Chapter 3 - Masking of Female SCN via Biosynthesis of Vanillic Acid in Engineered Soybean

Introduction to the soybean/SCN pathosystem

Soybean cyst nematode (*Heterodera glycines* Ichinohe, SCN) is an obligatory parasite of soybean and has the single largest impact on its production in the United States, causing more than a billion dollars in yield loss. This nematode causes more than twice the amount of yield loss than the next most important soybean pathogen (Allen, Bradley et al. 2017). In a particularly bad year, 2004, SCN caused over six million dollars in yield loss in Kansas alone (calculated) (USDA , Wrather and Koenning 2006, Council 2019). The parasite is found throughout the world where soybean is extensively cultivated. The first report of SCN in the United States was in 1954 in North Carolina, most likely coming from soil on root stock imported from Japan (Winstead, Skotland et al. 1995). Since its introduction SCN has spread throughout the United States wherever soybean is grown. Its spread is most likely due to movement of contaminated soil on form equipment, through wind, water, unclean seed, and in the digestive system of animals (Epps 1971). Early attempts to inoculate fields with growth promoting microbes by moving soil from one field to another most likely contributed to its spread as well. Although SCN is considered an obligate parasite evolving with soybean in Asia, there is debate as to whether SCN could be native to the United States, reproducing at low levels on weedy hosts (Riggs 1992). SCN belongs to the cyst nematode genus *Heterodera*. The nematode's current taxanomic classification is shown in Figure 3.1. Before its current description in 1952, SCN went by the scientific name *Heterodera schachii* for many years (CABI). There are currently 48 members of the *Heterodera* genus (Subbotin, Mundo-Ocampo et al. 2010). The most useful features for distinguishing between these species are the presence of males, stylet and tail

characteristics. Race and Hg-type characterization schemes have been used to describe SCN populations by assessing virulence on different indicator soybean lines (Riggs and Schmitt 1988, Niblack, Arelli et al. 2002). Populations of nematodes in fields can be quite diverse.

Domain	Eukaryota
Kingdom	Animalia
Phylum	Nematoda
Class	Chromadorea
Order	Tylenchida
Suborder	Tylenchina
Family	Heteroderidae
Genus	*Heterodera*
Specific epithet	*glycines*

Figure 3.1 Taxonomic classification of SCN

SCN reproduce best in course, dry soils (Koenning and Barker 1995). Gravid females from the previous generation are deposited in the soil as hard protective cysts. SCN go through four molts throughout their lifetime, similar to the molting of insects. The first molt happens while they are still in the egg. After this, vermiform nematodes are released into the soil as second-stage juveniles (J2). Among other things such as abiotic factors, egg hatching is induced by soybean root exudates and the J2 use these exudates to find host roots (Tefft and Bone 1985, Hu, You et al. 2017). At the root, nematodes use a protrusible needle-like stylet to make an opening through which they can enter. Once inside the roots, they set-up a feeding site at the vascular tissue known as a syncytium. The syncytium is formed by the degradation of surrounding cell walls and the fusion of a number of cells resulting in a large multinucleate cell. After setting up a feeding site the J2 will enlarge going through their remaining three molts to become adults. Adult females will remain at their feeding site and males become motile and leave the roots. Unlike nematodes that reproduce through parthenogenesis, male SCN are required for reproduction. Males use chemotaxis to find female SCN by following a pheromone

gradient through the soil. The primary pheromone attractant produced by SCN females was identified by Jaffe et al. (1989) as vanillic acid. Once impregnated, females turn from white to a brown lemon-shaped cyst. Cysts can contain anywhere from 200-600 eggs and can remain viable in the soil up to two years after their introduction (Jackson, Smith et al. 2005). The lifecycle of this nematode can be as short as 3-4 weeks depending on soil temperature (Young 1992). Symptoms of plants parasitized by SCN include stunting, yellowing, and low yields. SCN is also associated with other diseases. In combination with the fungal pathogen *Fusarium virguliforme*, SCN has a synergistic effect in causing sudden death syndrome (Xing and Westphal 2013).

Most commercial crops have shown to be inefficient hosts for SCN (Riggs 1992). Riggs (1987) showed that J2 race 3 (HG type 0) SCN do not penetrate roots of wheat and corn, but alfalfa and several other legume species were penetrated and slight development was observed. Wheat and corn are therefore considered to be good rotation crops to keep SCN populations under control. Unlike other members of the *Heterodera* genus, SCN has a broad host range. Riggs (1992) summarizes efficient hosts for SCN coming from 22 families of plant, many of which come from the fabaceae family. A number of weed species have been shown to be hosts for SCN; however, only field pennycress (*Thlaspi arvense*) and henbit (*Lamium amplexicaule*) have been shown to produce cysts in significant amounts compared to susceptible soybean varieties (Poromarto, Gramig et al. 2015). Susceptibility of less compatible hosts can be race or HG type specific similar to *G. max*. Fields that are allowed to go fallow need to be carefully monitored to prevent compatible hosts from growing and bolstering SCN populations.

Natural genetic resistance is currently the most common method for controlling SCN populations as chemical control is either economically infeasible or deemed too harmful for the environment and human health. Biological control of this parasite often has low efficacy or is too

variable for dependable control. As stated previously, SCN eggs remain viable in the soil for several years. This limits the efficacy of crop rotation for control of the nematode. The most common source of resistance comes from the *rhg1* quantitative trait locus (QTL). This QTL is a 31 kb locus found on chromosome 18 and is made up of four open reading frames (ORF). Three genes at the *rhg1* locus that are important for SCN resistance include an N-ethylmaleimide sensitive factor (NSF), an α-soluble NSF attachment protein (α-SNAP), and a protein of unknown function. All three of the genes are important for resistance as knockdown for any one results in a susceptible phenotype (Cook, Lee et al. 2012). Resistance derived from *rhg1* differs depending on copy number, coding sequence, and gene dosage (Cook, Bayless et al. 2014). The Williams 82 variety of soybean is susceptible to SCN and has one copy of *rhg1* while PI 548402 (also known as Peking) and PI 88788 (background for Fayette) are resistant to SCN and have three and ten copies of the *rhg1* QTL respectively (Cook, Lee et al. 2012). Increasing SCN resistance by increasing copy number or overexpression of *rhg1* genes would seem to be a feasible option for overcoming current resistance to *rhg1* by SCN; however, a recent study showed high expression of a particular *rhg1* gene resulted in cytotoxicity and cell death in plants (Bayless, Smith et al. 2016). Another factor that makes *rhg1* mediated virulence concerning is the large amount of acreage of soybean that uses it for defense against SCN. In 2001 more than 90% of soybean grown in United States depended on the *rhg1-b* haplotype derived from PI 88788 for SCN resistance (Concibido, Diers et al. 2004). Not surprising, a more recent study showed increasing SCN virulence on plants relying on this type of resistance (Howland, Monnig et al. 2018). Another source of SCN resistance comes from PI 548402, which has the *Rhg4* QTL in addition to *rhg1* (Meksem, Pantazopoulos et al. 2001). *Rhg4* in PI 548402 is necessary for SCN resistance and encodes a serine hydroxymethyltransferase (Liu, Kandoth et al. 2012).

Several closely related perennial species of Glycine show resistance to SCN of varying types. Barriers to interspecific crossing between perennial *Glycine* spp. and soybean prevents introduction of these novel sources of resistance (Wen, Yuan et al. 2017).

A number of biotechnological approaches that make use of host-derived resistance and overexpression of native *G. max* genes have been successfully used to control SCN. For example, Klink et al. (2009) and Li et al. (2010) exploited SCNs natural RNAi by expressing essential SCN housekeeping and reproductive genes in soybean as tandem inverted repeats (TIR). When feeding on soybean expressing these TIR, the SCN RNAi system silences expression of its own genes thereby reducing its fitness or reproductive success. As for overexpression of soybean genes for increased resistance, Lin et al. (2016) showed that overexpressing genes involved in plant defense pathways decreased reproductive success of SCN. Although these methods show promise for control of SCN, they have not been deployed because of variable results under field conditions.

Another approach for SCN resistance could be attempting to disrupt nematode mating by masking the sex pheromone vanillic acid (VA). In 1982, Rende et al. demonstrated attraction of male SCN to females through movement assays. Later research showed male movement towards two female chemicals of different solubilities (Bone 1986). Extensive work was done to identify these chemicals and in 1989 Jaffe et al. showed VA to attract males in the same manner as females. The culmination of this work showed VA to reduce SCN cyst numbers when applied to soil in alginate-clay prills under greenhouse conditions. This method of control had varying success, but reductions in cyst numbers were drastic in some cases. Another compound tested in this study that significantly reduced cyst numbers was syringic acid (Meyer and Huettel 1996). It is assumed that VA from the prills confused males, preventing them from finding females.

Application of VA infused prills to fields for SCN control would increase labor and reduce soybean profitability for farmers. We hypothesize that transgenic plants producing VA would have the same effect as VA prills while keeping labor input the same and represent a minor decrease in profitability due to increased seed cost.

Elements of vanillic acid biosynthesis in transgenic plants

Here we explore different aspects behind the chemistry and natural biosynthesis of VA in order to engineer its production in soybean. VA is a naturally occurring aromatic compound that is a common precursor and degradative product of vanillin, the common food flavoring compound. It is a monohydroxybenzoic acid having a methoxy group at position three (meta conformation), with a molecular formula of $C_8H_8O_4$ and MW of 168.15 grams mol^{-1} (syn: 4-hydroxy-3-methoxybenzoic acid; conjugate base: vanillate). VA is poorly soluble in pure water, 1-to-806 parts, but solubilizes well in alcohols. Although VA is a minor component of cured vanilla pods of several species of plant coming from the Vanilla genus, particularly *Vanilla planifolia*, these pods represent the largest natural source of the compound. Several other plants have been reported to produce VA including lower plants such as ferns and algae (Johansen, Wubshet et al. 2011, Mencherini, Picerno et al. 2011, Yang, Kondratyuk et al. 2011). There are number of studies that show VA being produced naturally by soybeans; however, these studies look at soybean meal and not the roots where the SCN lifecycle takes place (Arai, Suzuki et al. 1966, S Freitas, Alves da Silva et al. 2018). No biosynthetic pathway for VA or vanillin in soybean has been elucidated to date. It is possible the VA observed in soybean meal is produced by a common bacterial symbiont however, which is discussed in detail below (Hernandez, Garcia-Plazaola et al. 1999). Since vanillin is more economically important than VA, its

biosynthesis has been extensively studied. This work has serendipitously shed light on the biosynthesis of VA as well. Several biosynthetic pathways for these compounds have been suggested, but the general consensus is that vanillin and VA are derived from the phenylpropanoid pathway with ferulic acid as its precursor (Zenk 1965, Gallage, Hansen et al. 2014). It should be noted that the phenylpropanoid pathway is downstream of the shikimate pathway in plants. Due to its economic importance, a number engineered metabolic pathways have been developed for production of vanillin in various bacteria and fungi; however, synthetic production is still preferred because it is far cheaper. Engineered pathways in biological organisms have starting substrates such as ferulic acid, eugenol, lignin, and phenolic stilbenes while synthetic processes use coniferin, eugenol, lignin, guaiacol, and glyoxylic acid as substrates (reviewed in: Ramachandra Rao and Ravishankar 2000). As for previous attempts to bioengineer plants to produce VA and similar compounds, Mayer et al. (2001) introduced a bacterial enoyl-CoA hydratase into tobacco to produce vanillin directly from feruloyl-CoA. They detected a VA glucoside and VA glucose-ester as minor components of extracts from these plants. Like the pathway chosen for production of VA in this research, efficient production of VA from feruloyl-CoA, such as that reported by Mayer et al. (2001), would likely require the introduction of a second transgene for the enzymatic conversion of vanillin to VA.

The synthetic pathway to VA in this research (Figure 3.2) is modeled after work done by Hansen et al. (2009) in which VA was biosynthesized in different types of yeast. It was chosen because of its energetic efficiency and the bioavailability of the starting substrate, 3-dehydroshikimic acid (3-DHS; syn. 3-dehydroshikimate). 3-DHS is an intermediate in the shikimate pathway which is responsible for anabolism of aromatic amino-acids in plants. For plants the shikimate pathway is found predominantly in the chloroplast. Starting substrates that

are used in other engineered pathways such as ferulic acid, eugenol, lignin, and stilbenes are part of metabolic pathways that are downstream of the shikimate pathway and likely not as abundant as 3-DHS. Using substrates such as lignin and stilbenes would possibly leave plants more susceptible to pathogens as lignin deposition is an early defense mechanism in response to pathogens and stilbenes are primarily used as defense compounds in plants (Boerjan, Ralph et al. 2003, Chong, Poutaraud et al. 2009). Furthermore, eugenol is not known to be produced by soybean. These considerations lend to the rationale for using 3-DHS as a starting substrate for VA production in soybean.

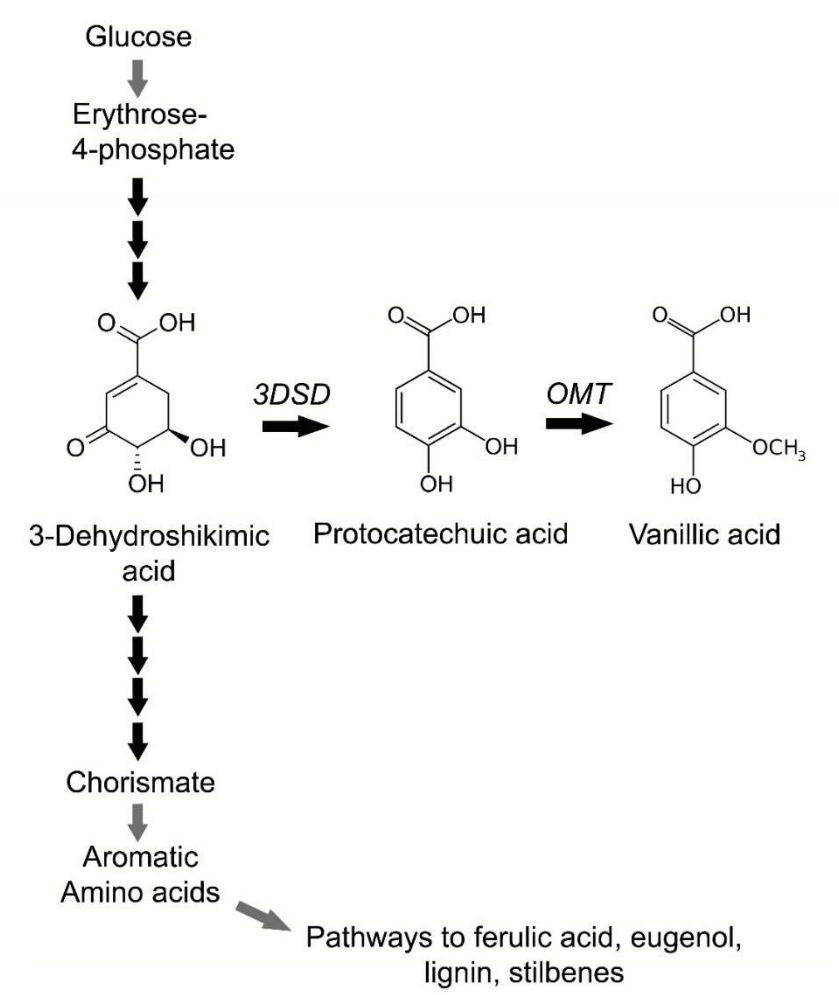


Figure 3.2 Hypothesized pathway to vanillic acid production in transgenic soybean via 3-dehydroshikimic acid.

Biosynthetic production of VA in soybean is predicted with the introduction of two transgenes, 3-dehydroshikimate dehydratase (*3DSD*) and an o-methyltransferase (*OMT*). Grey arrows represent multiple enzymatic steps. Black arrows represent a single enzymatic step. Other methods for biosynthetic production use downstream starting substrates ferulic acid, eugenol, lignin, and stilbenes.

To determine if this scheme has a reasonable chance for success, it is essential to consider other natural sources of VA found in the cropping systems that could already interfere with SCN reproduction. Several other field crops have been shown to produce this phenolic compound. Fields growing continuous sugarcane were shown to have VA concentrations in topsoil, as high

as 130 nmoles per 100g soil (Wang, Yang et al. 1967). Another source comes from the common mutualistic bacteria *Bradyrhizobium diazoefficiens* (*B. diazoefficiens*). This bacterium is commonly inoculated on soybean for their nitrogen fixing capacity. A complex interaction between *B. diazoefficiens* and *G. max* results in nodules on roots that are full of the bacteria. Hernandez et al. (1999) showed increasing levels of VA in plant leaves with increased amounts of the herbicide glyphosate. This is presumably due to the systemic nature of glyphosate, traveling to nodules and reducing EPSPS activity of the bacteria. This in turn shunts 3-dehydroshikimic acid away from aromatic AA production toward protocatechuic acid (PCA) and subsequently other derivatives such as VA. These compounds are then translocated to other parts of the plant. VA levels in glyphosate treated soybean reached as high as 366 nmoles per gram plant dry weight in their study and 1.3 μmoles per gram dry nodule. If glyphosate does indeed increase VA levels in plants which in-turn reduces SCN reproductive success as proposed in this thesis, one might expect application of glyphosate to be a suitable means for SCN control; however, glyphosate application has been shown to have no effect on SCN reproduction (Bradley, Noel et al. 2003).

A possible drawback of producing VA in plants is that it could have a negative effect on plant growth. Indeed, there have been several studies showing negative effects of VA on plants. At a concentration of 1mM, VA was shown to slow the speed of seed germination in *A. thaliana* (Reigosa and Malvido-Pazos 2007). In a soybean study, VA reduced dry weight, leaf area, and plant height of three-week-old plants at 1mM, but not 0.1 mM (Patterson 1981). This is probably higher than concentration of VA used in the SCN mating system as Jaffe et al. (1989) showed males react to concentrations of 0.1-10μM VA by coiling their bodies, a behavior similar to

when females are present. This group also reported that females produce only about 3.2pg of VA in a twenty-four-hour period.

3DSD

The first enzyme in the synthetic pathway used in this work is 3-dihydroshikimate dehydratase (EC:4.2.1.118; syn: DHS dehydratase; abbrev: 3DSD). 3DSD is found naturally in prokaryotes, fungi, and plants and catalyzes the conversion of 3DS to protocatechuic acid (syn. protocatechuate, 3,4-dihydroxybenzoic acid; abbrev: PCA). At least 176 homologs of 3DSD have been identified from a number of different microbes (KEGG pathways). The 3DSD CDS in this research comes from the ascomycete fungus *Podospora anserina* (*P. anserina*), a dung mold, which is part of the *P. anserina*/*P. pauciseta*/*P. comata* species complex (Boucher, Nguyen et al. 2017). This gene was chosen because of the previous success by Hansen et al. (2009) in using it to produce high levels of VA in several species of yeast (Saccharomyces spp.).

The enzymatic product of 3DSD and precursor to VA in this pathway is protocatechuic acid. Like VA, PCA is a benzoic acid with a hydroxyl group at position three. It has a molecular formula of $C_7H_6O_4$ and a MW of 154.12 grams mol^{-1}. PCA is soluble in 50 parts water. Alternative pathways to PCA in biological systems include a two-step process using the shikimate compound chorismate as a starting substrate as well as degradative pathways using vanillic acid, caffeic acid, and 4-hydroxybenzoic acid as immediate substrates (Kallscheuer, Vogt et al. 2016, Okai, Miyoshi et al. 2016). Although no biochemical pathway for the production of PCA has been elucidated for soybeans, plants susceptible to and treated with the shikimate pathway inhibiting compound glyphosate have been shown to accumulate high levels of PCA (Lydon and Duke 1988). Due to the lack of evidence for native *3DSD* genes in soybean,

it is unclear as to whether the elevated levels of PCA observed by Lydon and Duke were a result of metabolic processes of the plant, the mutualistic bacterium *B. diazoefficiens*, or catabolic processes. PCA has been shown to have very little effect on plant growth parameters compared to other phenolic compounds and is considered non-inhibitory (Shilling and Yoshikawa 1987). As for risks of increased levels of PCA to humans due to consumption of plants with increased levels, PCA is known to have a number of medicinal properties including being an antibacterial, antioxidant, antidiabetic, anticancer, anti-inflammatory, as well as other beneficial properties (reviewed in Kakkar and Souravh 2014).

OMT

O-methyltransferases (OMT) are found in a variety of organisms including animals and plants (Finkle and Kelly 1974, Bade, Christ et al. 1976). OMT's catalyze the methylation at oxygen groups for a variety of different substrates. For plants there are three main groups of OMT's depending on their substrates, caffeoyl CoA OMTs (CCoAOMT), carboxylic acid OMT's, and OMTs that methylate hydroxycinnamic acids, flavonoids, and alkaloids (Lam, Ibrahim et al. 2007). Plant OMT's can be substrate specific or fairly promiscuous (Ibrahim, De Luca et al. 1987, Chiron, Drouet et al. 2000). These enzymes are intricate in lignin formation and the production of defense compounds such as phytoalexins (Rakwal, Hasegawa et al. 1996, Pincon, Maury et al. 2001).

The OMT chosen for the synthetic pathway in this research comes from *Nicotiana tabacum*, Genebank accession AF484252.1, a class two OMT. Two classes and three different OMT enzyme activities have been identified in *N. tabacum*, each using *S*-adenosyl-L-methionine (SAM) as a methyl donor. The first class of OMT, NtOMT-I, is expressed in heathy tissues while the second class, NtOMT-II and III, show increased activity by tobacco mosaic virus infection.

Methylation resulting from these enzymes is fairly promiscuous, as Legrand et al. (1978) show they are capable of methylating at least thirteen different phenolic substrates. These authors also showed that NtOMT I preferentially methylates protocatechuic acid at the para position resulting in isovanillic acid while NtOMT-II and III methylate at the meta position resulting in VA. This is in line with what Hansen et al. (2009) observed in their attempt to produce vanillin in yeasts via recombinant OMT's from a variety of different sources. The NtOMT-I isoforms used in their experiments did not produce vanillic acid.

A number of methyltransferases have been identified in *G. max* including anthranilate, aspartate, catechol, CCoA, isoprenylcysteine, indole-3-acetate, jasmonate, and tocopherol OMTs; however, there is no evidence for vanillic acid production or enzymes characterized that methylate PCA at the meta position in soybean. Evidence for the lack of OMT's capable of converting PCA to VA can be seen from stable, low level concentrations of VA despite elevated PCA concentrations in experimental soybean (Lydon and Duke 1988). Phylogenetic analysis of native *G. max OMT*'s compared to the *N. tabacum* transgene used here along with other N. tabacum transgenes are shown in Figure 3.3. The work done for the research presented here investigates exogenous application of VA as proof-of-concept for GM soybean in reducing SCN numbers and produces several lines of soybean expressing transgenes for the enzymes discussed above for the production of VA *in planta*.

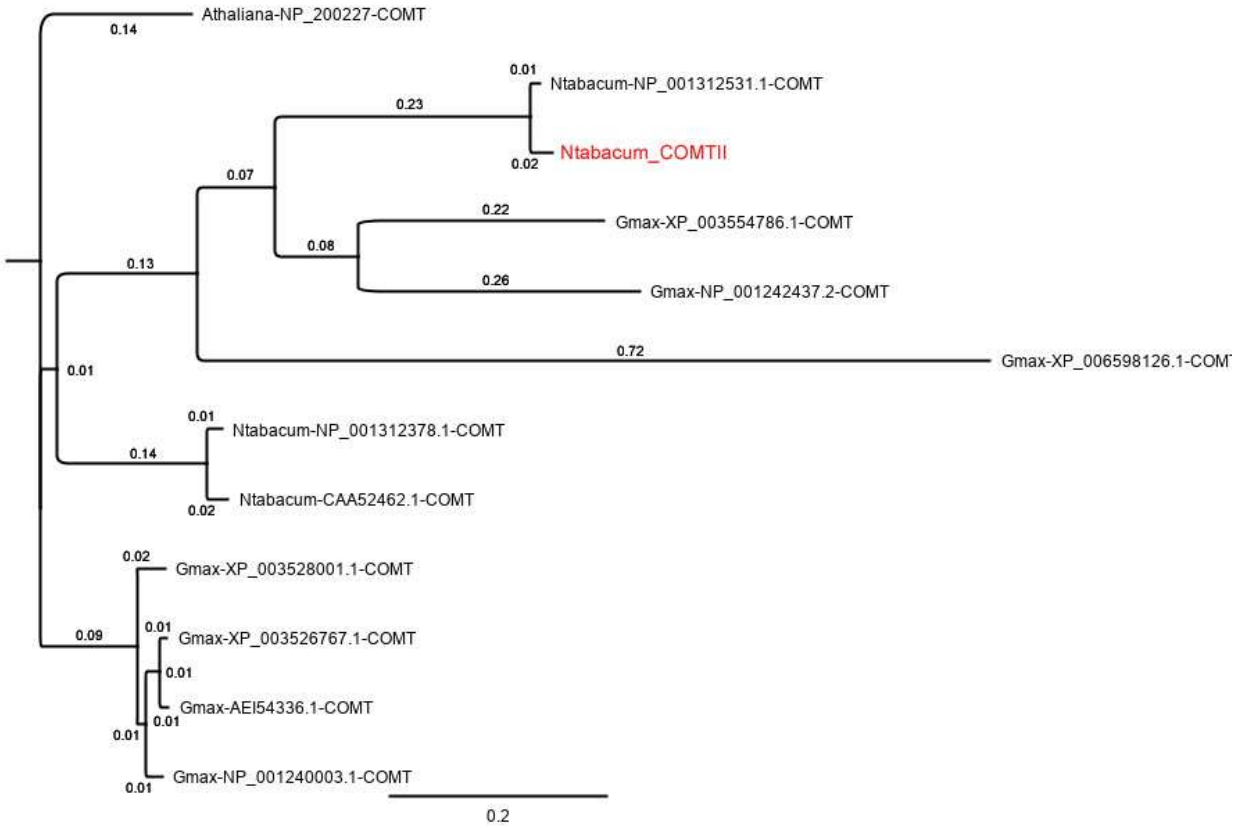


Figure 3.3 Phylogenetic analysis of *G. max* and *N. tabacum* OMT's

The transgene used in this work, shown in red, compared to other known tobacco and soybean OMT's as well as predicted OMTs from soybean with high sequence similarity. An *A. thaliana* OMT is used as an outgroup. Branch distances are indicated.

Materials and methods

Root length bioassays

To check for inhibition of VA on soybean root growth at physiological concentrations produced by female SCN, Williams 82 soybean seedlings where exogenously treated with different concentrations of VA. All seed used in bioassays were first sterilized with chlorine gas by placing an open petri dish with a monolayer of seed in a glass container. Chlorine gas was produced by mixing 3.3mL 12M HCl with 100mL 5.25% chlorine bleach. The glass container lid was closed, and seed was left to sterilize overnight. Seed sterilization was conducted in a

chemical fume hood. Seed was placed with sterile forceps into 165 × 180 mm CYG germination pouches (Mega-international; Newport, MN). Six seeds per pouch were planted with three replicates per treatment. Plants were grown in a Conviron (Winnipeg, MB) MTR30 growth chamber controlled by a CMP6050 control unit. Growth conditions for experiments were 16 hr day/8 hr night photoperiod at 26 ± 2°C and RH of 80%. Experimental treatments included 1, 30, and 1,000nM VA, as well as a modified ½ OMS treatment as a negative control. VA treatments were prepared from VA stock solution diluted with ½ OMS. A completely random design was employed in these experiments. Seeds were given 18mL of treatment solution initially and 10mL as needed thereafter to prevent desiccation. Plants where grown for 13 days post-emergence before harvesting. The least vigorous seedling per pouch was removed at harvesting and excluded from analysis. At harvesting, roots were removed from the rest of the plant at the crown. Roots were scanned on a flatbed scanner and total root length was calculated using the image analysis software APS Assess 2.0 (American Phytopathological Society; St. Paul, MN) (Figure 3.4).

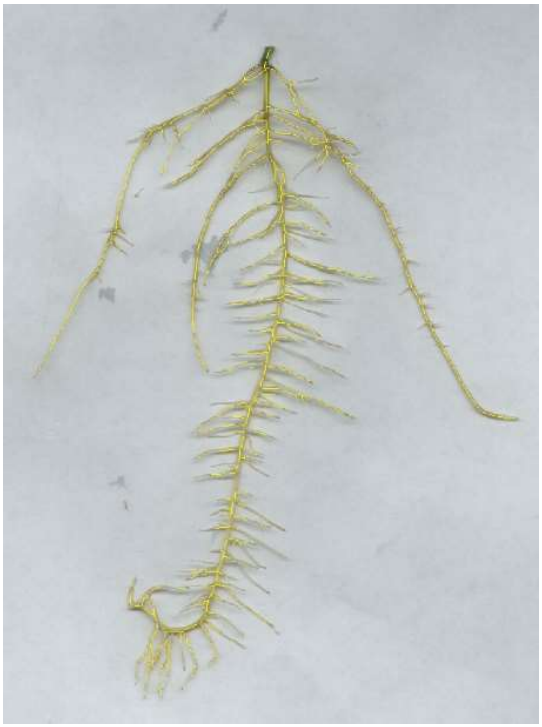


Figure 3.4 Root length analysis example of VA treated soybean
Example of APS Access 2.0 image analysis of total root length.

Exogenous VA SCN bioassays

As a proof-of-concept, soybean seedlings challenged with SCN were treated with exogenously applied VA solutions to check for reduced cyst and egg numbers. These experiments were carried out under greenhouse conditions at various times of the year with day/night temperatures of 27/22 ± 2°C, respectively. Natural light was augmented with 16 hr of sodium growth lamps. For experiment one a completely randomized design (CRD) was employed with one seed sown per tube. This was the experimental unit for experiment one Sample sizes ranged from 3-6. For experiment two and three, a randomized complete block design (RCBD) was used. Experiments two and three had a sample size of five to six. Three seeds were planted per tube and plants emerging from a single tube were treated as a single experimental unit. The SCN susceptible cultivar KS3406 was used for each experiment. Seed

was lightly coated with dry *Bradyrhizobium diazoefficiens* powder inoculum before sowing. Plants where grown in D40 Deepots (Stuewe and Sons; Tangent OR) containing a sand-soil mixture infested with SCN Hg type 7 population eggs. SCN inoculum was previously maintained on KS3406 soybean. Treatment solutions were made fresh at each watering from a VA stock solution and deionized water. Plants were watered as needed to prevent desiccation. Plants were harvested after 40 days post-emergence. Counts where normalized against total grams dry root mass (GDRM) per pot. Roots were dried in the dark in a drying room at ~60°C for 48 hrs. SCN females, cysts, eggs, and J2 were harvested and quantified as described by Brady et al. (2012).

Development of recombinant *3DSD* & *COMT* soybean

Transgenic soybean lines expressing previously described 3DSD and COMT CDSs for predicted VA production were generated. After codon optimization genes were synthesized and cloned into two separate plasmids with pUC57 backbone by Genescript. Overexpression of these transgenes in *G. max* was accomplished with the pGmubi vector (Figure 3.5). This plasmid is 4,586 bp in size. VA CDSs were directionally sub-cloned into this vector through *Bam*HI and *Not*I double restriction digestion followed by T4 ligation, which subsequently removed the native sGFP(S65T) reporter gene. Gene expression from this plasmid is driven by a constitutive *G. max* polyubiquitin promoter and terminated by Tnos. The plasmid also carries *bla* for ampicillin resistance to aid in bacterial sub-cloning (Chiera, Bouchard et al. 2007). For positive selection of *G. max* calli, pGmubi constructs were co-bombarded with the pHyg plasmid. This plasmid confers resistance to the antibiotic hygromycin via a hygromycin B phosphotransferase gene (*hph*) which is expressed in plants by a CaMV35S promoter and terminated by Tnos. Both soybean vectors, pGmubi and pHyg, were developed by the same research group (Finer, Vain et al. 1992, Chiera, Bouchard et al. 2007). A biolistic and tissue culture transformation scheme

developed by Trick et al. (1997: Protocols 3 & 5) was used for regenerating transgenic plants from soybean calli.

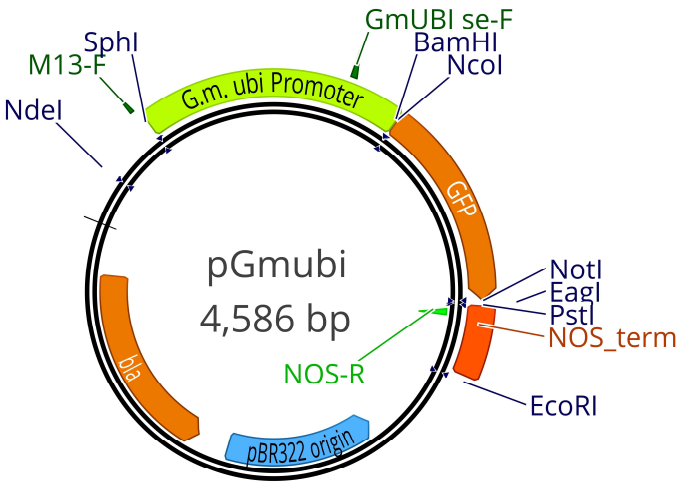


Figure 3.5 Diagram of pGmubi plasmid

Annotations include *Glycine max* ubiquitin promoter (G.m. ubi Promoter), synthetic green fluorescent protein (GFP), nopaline synthase terminator (NOS_term), pBR322 origin of replication, ampicillin resistant gene (bla), forward (*-F) and reverse (*-R) primer binding sites, and unique restriction sites.

SCN challenge of VA transgene expressing soybean

Soybean transformed via particle bombardment and expressing genes predicted to produce VA were challenged with SCN under greenhouse conditions as described in the exogenous VA-SCN bioassay section. The transformant background JackX was used as the negative control. A CRD was used for these two experiments. Because harvesting SCN for SCN bioassays is destructive for the plant and T_1 seed was limited, sample sizes were small for these assays. Twelve seed were sown for each experimental treatment (Ghast5, Ggauze1, and Ggauze4) and six for each control treatment. Plants not expressing transgenes were removed

from statistical analysis as well as stunted plants with GDRM below 0.08. Cysts were harvested from soybean and quantified as described by (Brady, Li et al. 2012).

Developing synthetic VA expression operon for *Bradyrhizobium diazoefficiens*

The mutualistic bacterium *Bradyrhizobium diazoefficiens* (*B. diazoefficiens*) is commonly inoculated on soybean for its nitrogen fixing capacity. After a well-choreographed interaction between plant and bacterium, a novel organ forms on roots in which *B. diazoefficiens* is maintained and provides atmospherically fixed nitrogen for the plant. Because transformation of bacteria normally takes days to weeks compared to years for soybean, a plasmid was developed for expression of 3DSD and COMT CDSs for this bacterium as an alternate route for VA production in soybean.

Development of this plasmid was modeled after successful expression of recombinant green fluorescent protein (GFP) in the closely related *Rhizobium leguminosarum* by Stuurman et al. (2000). Stability of this plasmid under non-selective conditions is conferred by stability elements found on the plasmid backbone pME6010 (Figure 3.6) (Heeb, Itoh et al. 2000). The p15A replicon containing *oriT* on this plasmid also allows for conjugation when used in the appropriate host strain. The process for developing this plasmid, p6010VA, is shown in Figure 3.7 and has been sequence-verified. Competent cells for electroporating *B. diazoefficiens* were made according to Hattermann and Stacey (1990). As an alternative to electroporation, *E. coli* strain S17 was used for conjugation transformation of *B. diazoefficiens*. Conjugation was performed as described by Hahn and Hennecke (1984) with modified arabinose gluconate (MAG) media substituted for *B. diazoefficiens* growth media.

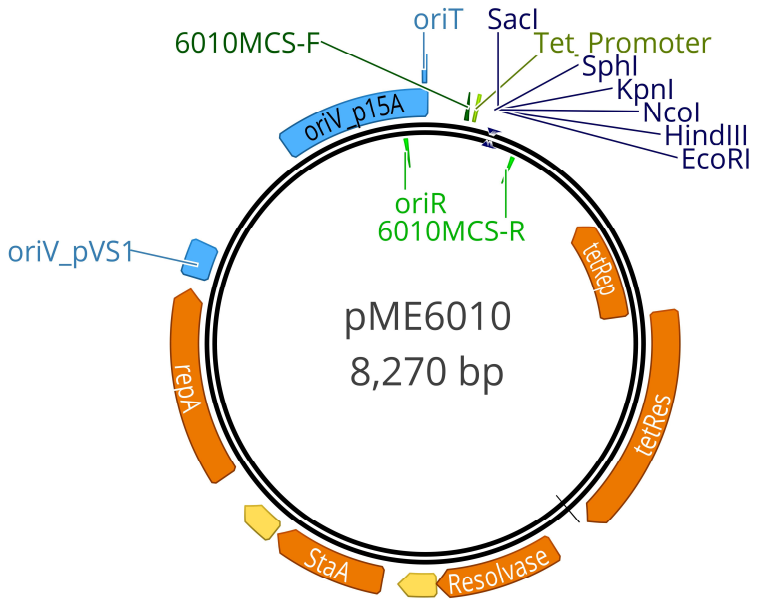


Figure 3.6 Diagram for pME6010 plasmid

Annotations include tetracycline resistance promoter (Tet Promoter), pVS1 and p15A origin of replications, origin of transfer (oriT) tetracycline resistant gene (tetRes), tetracycline repressor (tetRep), an ORF for a segregation stability factor (staA), a predicted resolvase, an ORF predicted to function in plasmid replication (repA), forward (*-F) and reverse (*-R) primer binding sites, and unique restriction sites.

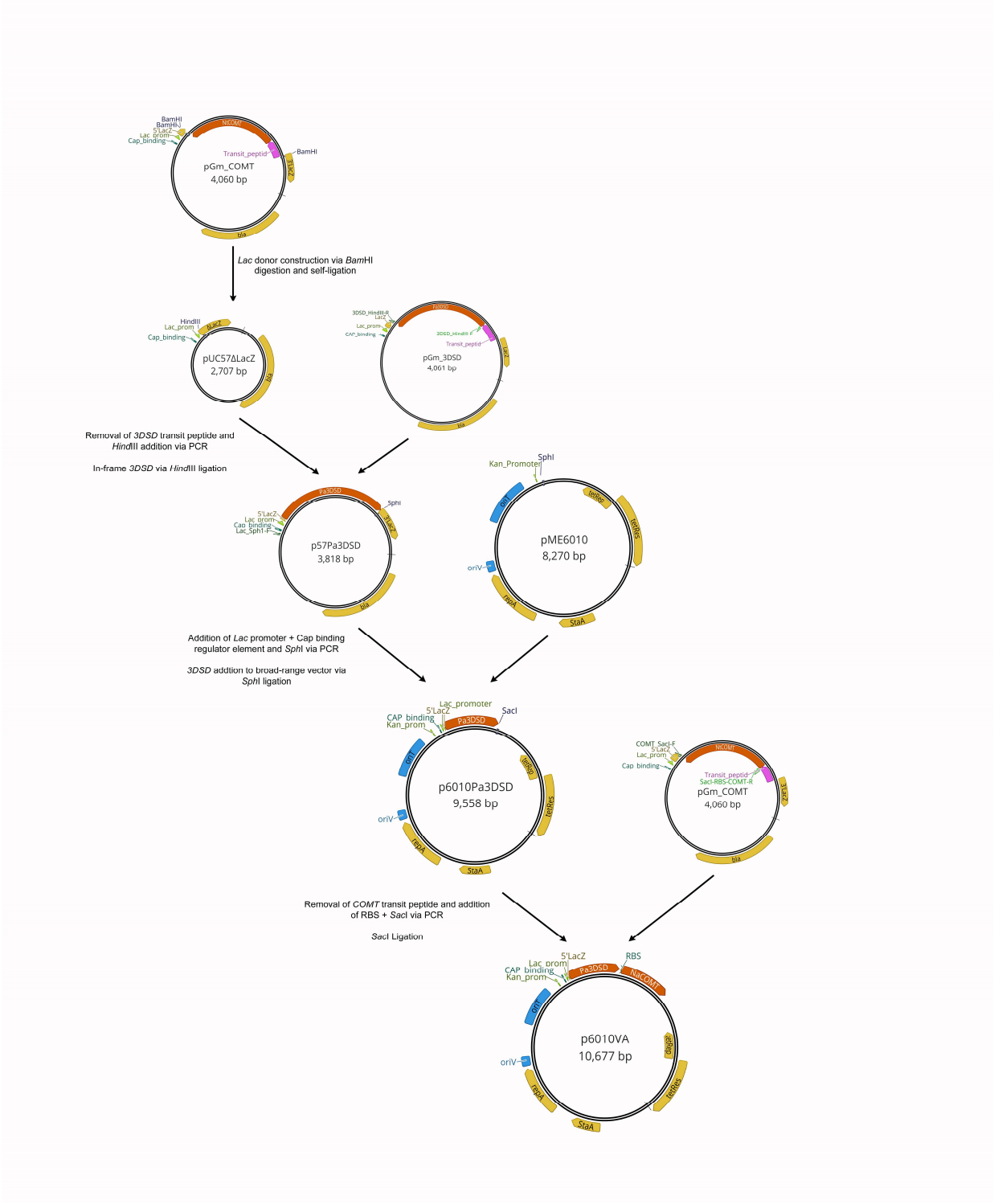
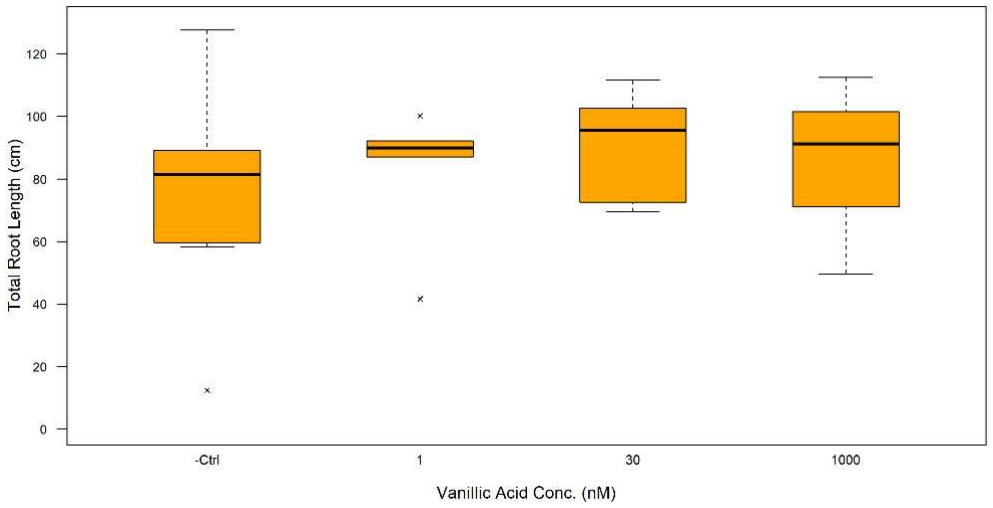


Figure 3.7 Schematic for construction of synthetic 3DSD-COMT operon for expression in *Bradyrhizobium diazoefficiens*

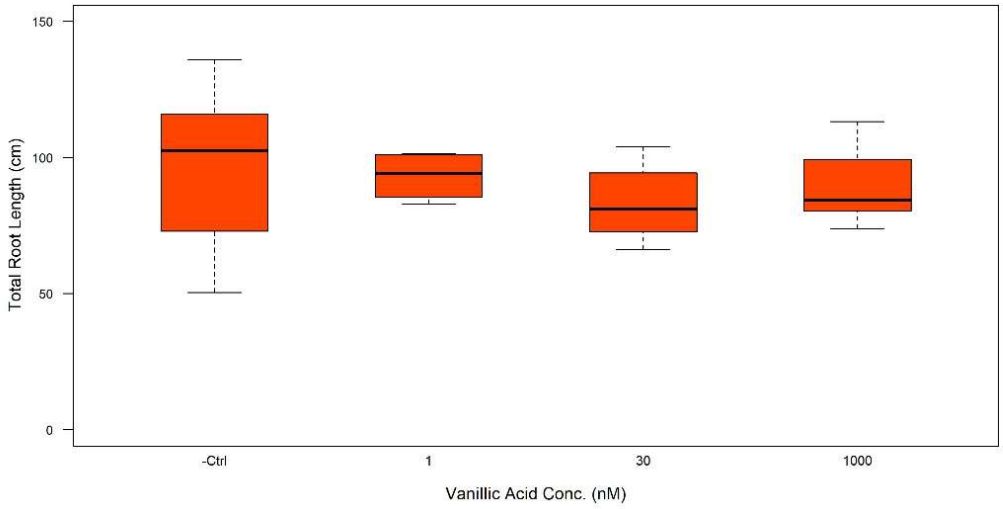
Results and discussion

Root length bioassays

For the root length bioassays checking for inhibition of VA on total root length, mean root lengths for experimental treatments were higher than the negative control group in experiment one and lower than the negative control group in experiment two (Figure 3.8). A Dunnett's test did not show significant differences for experimental groups against the control ($p < 0.05$). The smallest adjusted p values were 0.617 and 0.621 for experiments one and two respectively. The lack of a trend in means between experimental groups and the control across the two experiments could signify a balance between negative effects seen by Patterson et al. (1981) and growth promoting potential due to an extra source of carbon. Mean root lengths between the two experiments showed no significant difference (ANOVA p value = 0.27). These experiments show conceptually, transgenic soybeans producing VA at levels used by SCN would not inhibit plants themselves; although, this does not account for possible effects of intracellular presence of VA in such plants.



A.



B.

Figure 3.8 Soybean root length inhibition assays treated with VA.

Graphs showing total root lengths (cm) for soybeans treated with various concentrations of VA (nM) against a negative control (-Ctrl) for bioassays one (A) and two (B).

Soybean/SCN VA watering bioassays

For SCN-VA watering bioassays mean cyst numbers where generally higher for experimental treatments compared to the negative control treatment (Figure 3.9 & Figure 3.10). Exceptions to this were the 30 and 1000nM pretreatment watering schemes in bioassay one and the 30nM treatment for bioassay two. No significant differences were found for experiments one or two when evaluating cyst and egg numbers using ANOVA, *p* values 0.62 and 0.24 for cysts

and eggs respectively for experiment one and 0.35 and 0.67 for cysts and eggs respectively for experiment two. There was also no interaction between VA concentration and watering scheme for bioassay one (p value > 0.05 for both cyst and eggs). For bioassay three a post-hoc Dunnett's multiple comparisons test (Dunnett's test) showed significant differences between 30nM, 1000nM, and 2000nM treatments for cysts per GDRM compared to the negative control ($p < 0.05$); however, a Tukey's HSD test only showed a significant difference in cysts per GDRM for the 30nM treatment group. Similarly, a Dunnett's test showed a significant difference between 30 and 2000nM treatments and the negative control for eggs per GDRM ($p < 0.05$), while a Tukey's HSD test showed no significant differences.

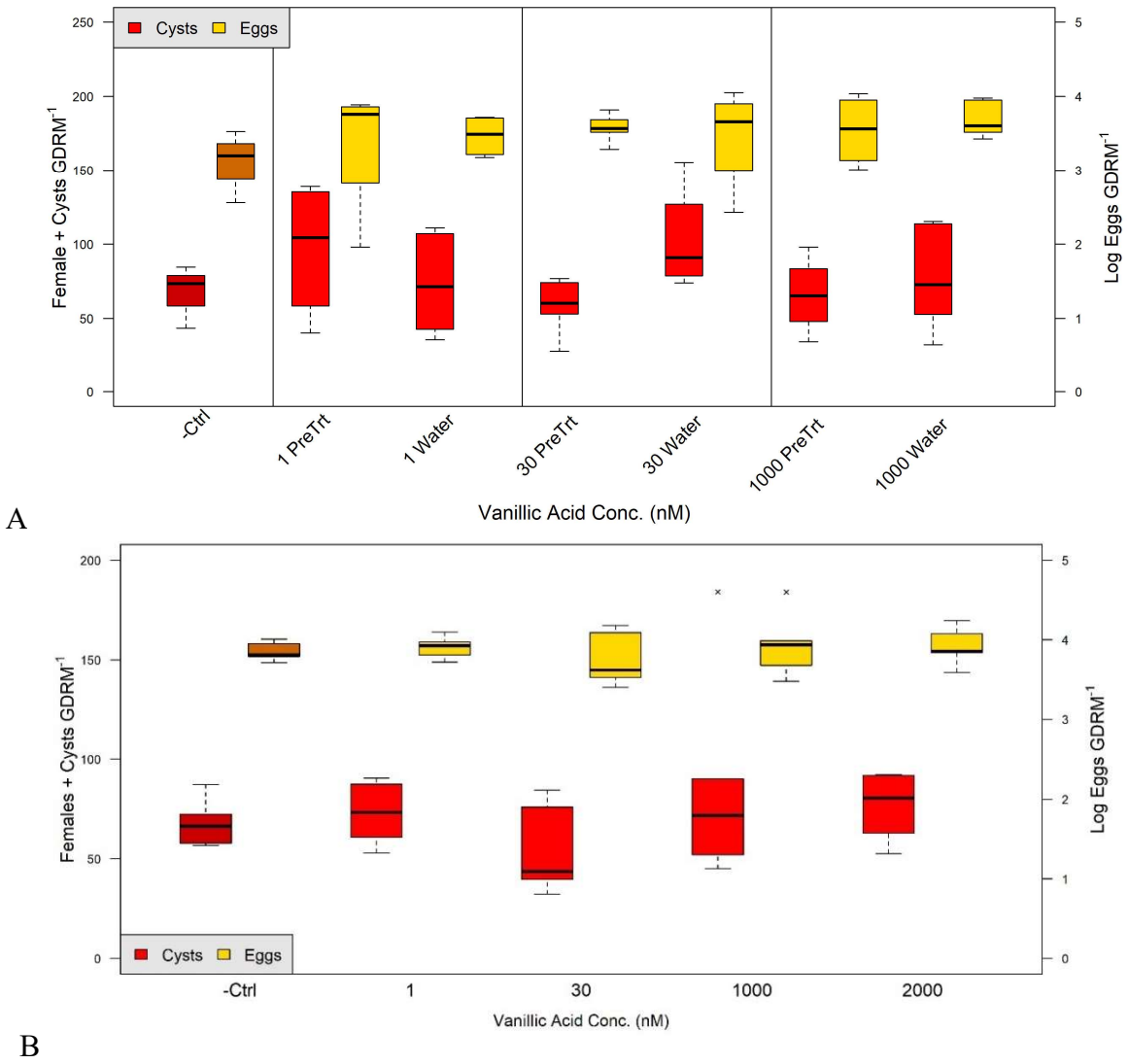


Figure 3.9 Effects of exogenously applied VA on SCN cyst and egg numbers per gram dry root mass bioassays one and two.

Soil for pretreatments (PreTrt) in experiment one (A) were mixed with VA solutions indicated as well as irrigated with corresponding VA solution, while water only treatments (water) were irrigated with VA solution. The negative control treatments were irrigated with tap water. Experiment two used water only type experimental treatments. No significant differences were found between experimental treatments and negative controls for either cyst or eggs $GDRM^{-1}$ for either experiment with a Dunnett-test. A nested ANOVA showed no effect of VA concentration on cyst and egg numbers and no interaction between VA concentration and watering regime (p values > 0.05).

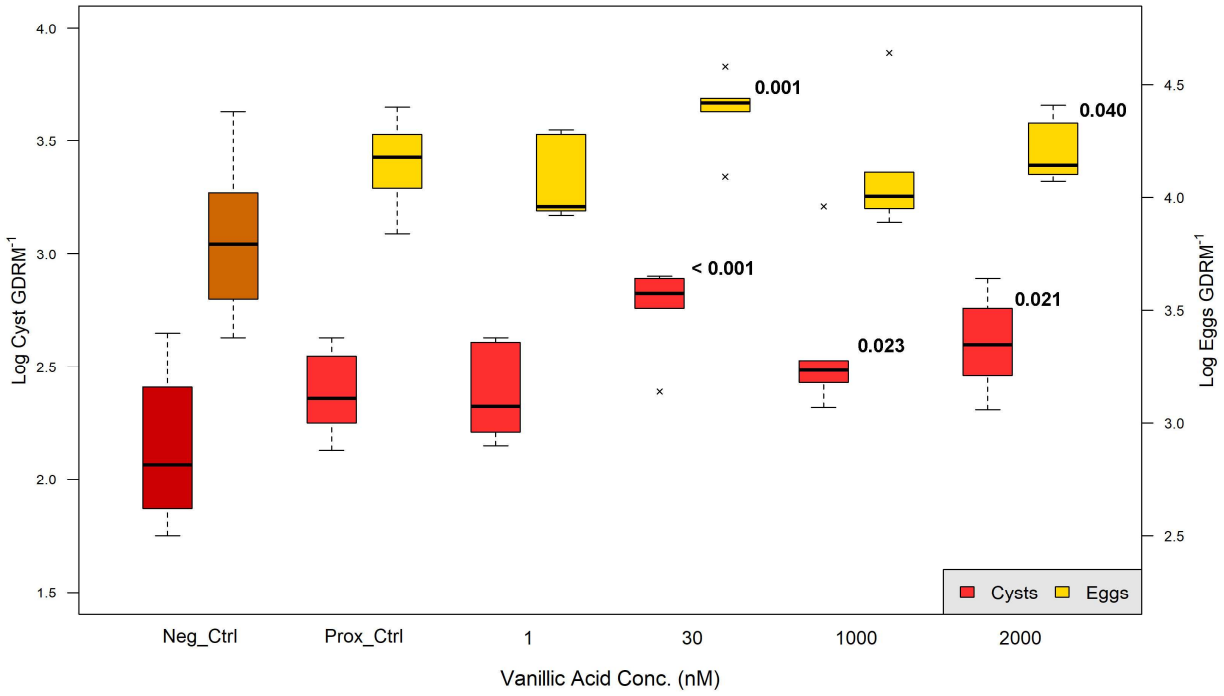


Figure 3.10 Effects of exogenously applied VA on SCN cyst and egg numbers per gram dry root mass bioassay three.

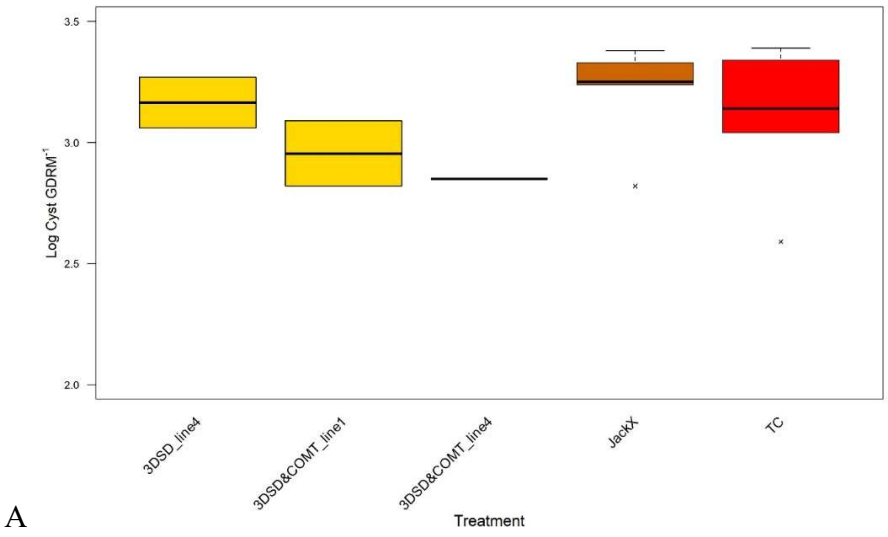
Plants in the negative control group (Neg_Ctrl) received tap water only. Plants in the proximity control group (Prox_Ctrl) were grown in an adjacent greenhouse to rule out hormonal signaling between plants and aromatic contamination. Boxes accompanied by numbers (*p* values) are significantly different compared to the negative control via Dunnett's test.

Overall, there was an upward trend in these three experiments for egg and cyst production on soybean roots from experimental groups. This could nullify the theory that transgenic soybean engineered to produce VA would reduce reproductive success of SCN, but instead may actually exacerbate the problem. Like other bioassays, good infection of a susceptible control is often required for an experiment to elucidate significant differences between treatments. Lower mean cyst numbers in bioassays one and two compared to bioassay three may give stronger weight to results in bioassay three. The different application methods of

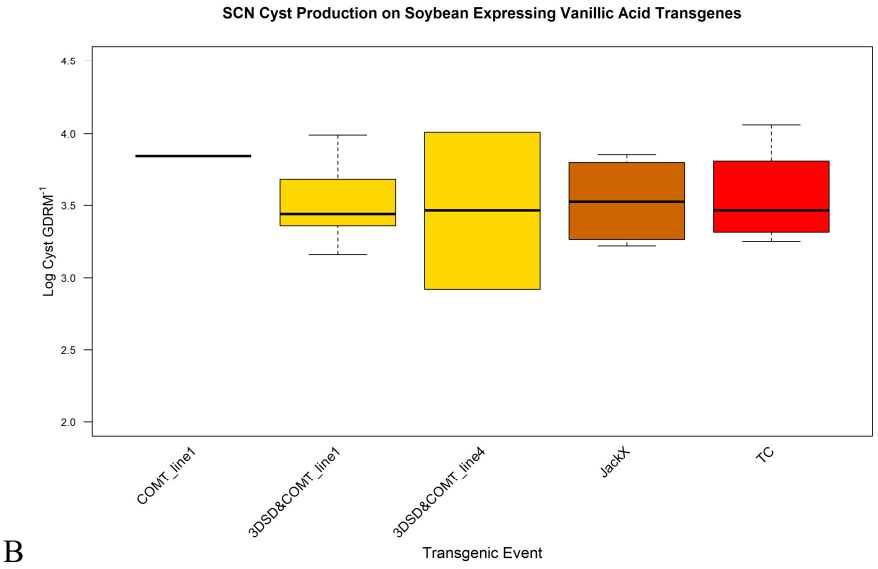
VA between this study, exogenous treatment through VA irrigation, and the prill-type method used by Meyer et al. (1996) may account for the difference seen in effects between the two. The prill method could better simulate SCN females producing a concentration gradient of VA for males to follow away from females, while the watering method would likely produce a uniform concentration of VA throughout the soil.

Transgenic 3DSD and COMT soybean/SCN bioassays

For the bioassays with transgenic soybean challenged with SCN, sample sizes for controls ranged from 4-5. Sample sizes for experimental treatments were small due to limited amount of T_1 seed and plants lacking transgenes due to independent assortment or transgene silencing were removed from statistical analysis. Experimental treatments ranged from 1-4. Lower cyst numbers on experimental groups would support our prediction that then introduced transgenes would produce VA in soybean. Means for experimental treatments in experiment one were lower than the JackX W/T control while there was no clear pattern in experiment two (Figure 3.11). Mean cyst numbers per GDRM for plants expressing a single transgene were higher than plants expressing both transgenes in both experiments. ANOVA's for both experiments showed no significant differences among treatments in either experiment (smallest *p* value 0.65).



A



B

Figure 3.11 SCN cyst numbers on transgenic vanillic acid soybean lines bioassays.

Log cyst numbers per GDRM on lines expressing different combination of transgenes for bioassays one (A) and two (B). Transgene expression by Ggauze line given. Negative controls are background JackX variety and tissue culture (TC) control

To determine definitively if plants expressing *3DSD* and *COMT* effect SCN cyst and egg production, experiments with a larger sample size needs to be performed. More lines could be

developed and assayed as well. Another aspect that was not evaluated here is whether these plants are actually producing VA. Several qualitative and quantitative schemes for VA have been developed. Jaffe et al. (1989) used an extensive, four-step HPLC procedure to identify VA from SCN females. More recently, simpler methods have been developed for detection of VA in plant materials (reviewed in: Stalikas 2007). Alternatively, a biosensor system can be used for quantifying VA concentrations from soybean cell extracts. For example, a VA specific biosensor system was developed by Kunjapur & Prather (2019) in which dose-dependent expression of a fluorescent protein is driven by the presence of VA in *E. coli*.

A number of reasons might account for the absence of VA in the transgenic soybean developed for these experiments. Plants evaluated here were shown to express *3DSD* and *COMT*. This does not account for the presence of the proteins themselves or their functionality in the host cell. The three-dimensional confirmation of the proteins may be altered due to differences in physiology or protein processing pathways compared to their native system. The subcellular location of both proteins in their native systems is not known and neither have predicted chloroplast targeting sequences; therefore, since this system targets the recombinant proteins to the chloroplast, its altered pH from the cytosol may reduce their activity or render them nonfunctional. Being recombinant proteins, other factors in plant cells may be involved in reducing their activity. The proteins could fail to localize to the chloroplast, fail fold properly, or be marked for degradation soon after translation. Although there is no information on glycosylation of these proteins in their native species, this could deviate as well. A straightforward way to check for functionality of the recombinant proteins from the soybean system would be to treat substates for the enzymes with protein extracts and check for product formation. Also, *3DSD* and *COMT* could be introduced into other plant systems to validate their

activity. Work has also been done in this research for introducing these transgenes into *A. thaliana* through *Agrobacterium*-mediated transformation. *3DSD* and *COMT* transgenes sub-cloned into the pBICaMV binary vector have been developed along with four lines of *A. thaliana* with recombinant *3DSD* that await to be characterized.

Low levels of VA may also be due to lack of the OMT cofactor S-adenosyl-L-methionine (SAM). Overexpression of enzymes responsible for the biogenesis of SAM could also help to increase VA production in transgenic soybean. It should be noted that SCN produces a chorismate mutase that is believed to perturb the shikimate pathway in soybean (Bekal, Niblack et al. 2003). This perturbance is thought to increase susceptibility of the soybean by reducing the abundance of defense compounds such as indole-3-acetic acid, salicylic acid, and phenolic phytoalexins. This would most likely not affect VA levels in transgenic plants as 3DS is upstream of chorismate. Alas, even if these plants are shown to produce VA, their inability to reduce SCN reproductive success through masking of females cannot be ruled out because of non-efficacy of VA. It is possible VA is not secreted from plant roots into the rhizosphere, the mating court for SCN. Secretion of VA could require introduction of specific recombinant transporters. Although none seem to be identified for exporting VA, there is evidence of transporters in fungi for the uptake of VA (Shimizu, Kobayashi et al. 2005). Another strategy could be to transform soybean with protocatechuic acid chloroplast transporters for conversion to VA in the cytoplasm.

Genetic characterization of recombinant 3DSD and COMT soybean

In order to generate transgenic soybean predicted to produce VA more than 1,000 soybean calli were bombarded with the *3DSD* recombinant gene, 800 calli with *OMT*, and more

than 2,500 calli for combined bombardment with *3DSD* and *COMT*. From these bombardments one plant was regenerated and fertile harboring *3DSD*, four for *COMT*, and four for both *3DSD* and *COMT* transgenes. Several of these lines were characterized at the T1 generation for bioassays and seed bulking (Table 3.1). Of these, plants from the single *COMT* only line Ghast5 had no transgene expression. Four lines with both *3DSD* and *COMT* showed similar segregation between lines at the T_1 generation. Segregation between the transgenes *3DSD* and *COMT* was the same for lines Getaway4, Gguaze1, and Ggauze4, while *COMT* was found in fewer plants than *3DSD* for line Getaway5. The occurrence of expression for *3DSD* was higher than *COMT* for lines Getaway4, Ggauze1, and Ggauze4, while line Getaway5 had a higher occurrence for expressing *COMT* compared to *3DSD*.

Line	GOI	Obs.	Pos. GOI	% GOI pos.	Exp. Obs.	Pos. Exp.	% Pos Exp.
Ghast5	*COMT*	18	12	66.7%	12	0	0.0%
Getaway4	*3DSD*	18	8	44.4%	6	4	66.7%
	COMT	18	8	44.4%	6	1	16.7%
Getaway5	*3DSD*	12	6	50.0%	5	4	80.0%
	COMT	12	5	41.7%	4	4	100.0%
Ggauze1	*3DSD*	35	24	68.6%	21	13	61.9%
	COMT	35	24	68.6%	21	10	47.6%
Ggauze4	*3DSD*	31	20	64.5%	18	9	50.0%
	COMT	31	20	64.5%	18	8	44.4%

Table 3.1 Segregation and expression information for T_1 VA soybean lines

Abbreviations include gene of interest (GOI), observed individuals (Obs.), observed individuals for expression analysis (Exp. Obs.), positive (pos.), catechol o-methyltransferase (COMD), and 3-dehydroshikimate dehydratase (3DSD)

Transformation of *Bradyrhizobium diazoefficiens* for VA production

Transformation of *B. diazoefficiens* was not successful with p6010VA by electroporation or conjugation. In addition to pME6010 and its derivatives, variants of the broad host range vector pBBR1MCS with a variety of selectable marks were used in electroporating *B. diazoefficiens* (Kovach, Elzer et al. 1995). Nine electroporation attempts with various plasmids across four batches of competent cells were performed. Despite these attempts, colony PCR revealed no successful transformation events.

A number of things can be tried for successful transformation of *B. diazoefficiens* with this plasmid. It has been shown that plasmids coming from *E. coli* with reduced methylation activity, genotype *dam*$^{-}$ and *dcm*$^{-}$, allow *B. diazoefficiens* to be transformed at an order of magnitude higher compared W/T (Guerinot, Morisseau et al. 1990). Plasmids produced for these studies came from DH5α which is *dam*$^{+}$ and *dcm*$^{+}$. This could be altered in the future to increase chances of successful transformation by using stains such as GM2198 and GM2163 for plasmid preparation (Marinus, Carraway et al. 1983, Guerinot, Morisseau et al. 1990). Also, conjugation is generally considered to be more efficient in transforming recalcitrant Gram-negative bacteria compared to electroporation. More attempts can be made at conjugation transformation. Simpler transformation techniques can also be tried. Although several orders of magnitude less efficient than conjugation or electroporation, a freeze-thaw method for *Bradyrhizobium* spp. has shown to be successful (Vincze and Bowra 2006).

In general, VA alone and plants hypothesized to produce VA did not reduce successful mating of SCN as hypothesized. This leads one to question if VA is actually a sex pheromone for SCN. Indeed, work on male SCN coiling behavior in the presence of females and VA separately was repeated in this research with no clear results (Huettel and Rebois 1986, Jaffe, Huettel et al.

1989: data not presented). Attraction type assays, such as those performed by Huettel & Rebois (1986) and Jaffe et al. (1989), with purified VA and analogs needs to be done to validate VA as a sex pheromone. Along with these chemicals, known nematode pheromones such as ascarosides can be tested (Choe, von Reuss et al. 2012). Evidence of these compounds leads one to wonder if the attractant purified by Jaffe et al (1989) was actually an ascaroside instead of VA. Compared to other nematodes, SCN produces very little ascarosides (Manosalva, Manohar et al. 2015). In fact, Choe et al. (2012) showed no detectable production of ascarosides in a close relative of SCN, *Pratylenchus penetrans*, while Manosalva et al. (2015; supp. mtrl.) showed trace amounts of a single ascaroside being produced by *Pratylenchus brachyurus* and SCN. It is unlikely the native glycolipid ascaroside detected by Manosalva et al. (ascr#18) would give the same biochemical signature as VA under the procedures done by Jaffe et al. (1989) as it has a much higher MW and most likely different hydrophobicity properties; however, this could be a possibility with a degraded form of ascr#18. Results from the VA watering bioassays suggest that VA may worsen this problem. It is possible that VA may worsen cyst and egg production by acting as a hatching factor, although there is no evidence of this, and known hatching factors do not appear to be structural analogs of VA. (Masamune, Anetai et al. 1982, Nonaka, Katsuyama et al. 2016). VA may also act as a stimulant for SCN, increasing the activity of males once they emerge from the roots. No clear trends for SCN bioassays done with soybean expressing *3DSD* and *COMT* confound this prediction. Validation of VA production by transgenic plants, and challenging these plants against SCN in larger bioassays could answer a number of interesting questions raised by this research.

Works Cited

Allen, T. W., C. A. Bradley, A. J. Sisson, E. Byamukama, M. I. Chilvers, C. M. Coker, A. A. Collins, J. P. Damicone, A. E. Dorrance, N. S. Dufault, P. D. Esker, T. R. Faske, L. J. Giesler, A. P. Grybauskas, D. E. Hershman, C. A. Hollier, T. Isakeit, D. J. Jardine, H. M. Kelly, R. C. Kemerait, N. M. Kleczewski, S. R. Koenning, J. E. Kurle, D. K. Malvick, S. G. Markell, H. L. Mehl, D. S. Mueller, J. D. Mueller, R. P. Mulrooney, B. D. Nelson, M. A. Newman, L. Osborne, C. Overstreet, G. B. Padgett, P. M. Phipps, P. P. Price, E. J. Sikora, D. L. Smith, T. N. Spurlock, C. A. Tande, A. U. Tenuta, K. A. Wise and A. J. Wrather (2017). "Soybean yield loss estimates due to diseases in the united states and ontario, canada, from 2010 to 2014." Plant Health Progress **18**: 19-27.

Arai, S., H. Suzuki, M. Fujimaki and Y. Sakurai (1966). "Studies on flavor components in soybean; part ii: Phenolic acids in defatted soybean flour." Agricultural and Biological Chemistry **30**(3): 364-369.

Bade, P., W. Christ, D. Rakow and H. Coper (1976). "Comparison of catechol-q-methyltransferase from rat brain, erythrocytes and liver." Life Sci **19**(12): 1833-1844.

Bayless, A. M., J. M. Smith, J. Song, P. H. McMinn, A. Teillet, B. K. August and A. F. Bent (2016). "Disease resistance through impairment of alpha-snap-nsf interaction and vesicular trafficking by soybean rhg1." Proc Natl Acad Sci U S A **113**(47): E7375-e7382.

Bekal, S., T. L. Niblack and K. N. Lambert (2003). "A chorismate mutase from the soybean cyst nematode *heterodera glycines* shows polymorphisms that correlate with virulence." Mol Plant Microbe Interact **16**(5): 439-446.

Boerjan, W., J. Ralph and M. Baucher (2003). "Lignin biosynthesis." Annu Rev Plant Biol **54**.

Bone, L. W. (1986). "Fractionation of the female's pheromone of the soybean cyst nematode, *heterodera glycines*." The Helminthological Society of Washington **53**: 132-134.

Boucher, C., T.-S. Nguyen and P. Silar (2017). "Species delimitation in the *podospora anserina/ p. Pauciseta/p. Comata* species complex (sordariales)." Cryptogamie, Mycologie **38**(4): 485-506, 422.

Bradley, C. A., G. R. Noel, C. R. Grau, J. M. Gaska, N. C. Kurtzweil, A. E. Macguidwin, L. M. Wax, G. L. Hartman and W. L. Pedersen (2003). "Impact of herbicides on *heterodera glycines* susceptible and resistant soybean cultivars." J Nematol **35**(1): 88-97.

Brady, C. R., J. L. Li, T. C. Todd, T. R. Oakley and H. N. Trick (2012) "Compatibility of foliar insecticides and soybean cyst nematode bioassays." Plant Health Progress **Online** DOI: 10.1094/PHP-2012-0409-01-BR.

CABI. "Invasive species compendium; *heterodera glycines* (soybean cyst nematode) datasheet." Retrieved 9.11.19, from https://www.cabi.org/isc/datasheet/27027.

Chiera, J. M., R. A. Bouchard, S. L. Dorsey, E. Park, M. T. Buenrostro-Nava, P. P. Ling and J. J. Finer (2007). "Isolation of two highly active soybean (*glycine max* (l.) merr.) promoters

and their characterization using a new automated image collection and analysis system." Plant Cell Reports **26**(9): 1501-1509.

Chiron, H., A. Drouet, A. C. Claudot, C. Eckerskorn, M. Trost, W. Heller, D. Ernst and H. Sandermann, Jr. (2000). "Molecular cloning and functional expression of a stress-induced multifunctional o-methyltransferase with pinosylvin methyltransferase activity from scots pine (pinus sylvestris l.)." Plant Mol Biol **44**(6): 733-745.

Choe, A., S. H. von Reuss, D. Kogan, R. B. Gasser, E. G. Platzer, F. C. Schroeder and P. W. Sternberg (2012). "Ascaroside signaling is widely conserved among nematodes." Curr Biol **22**(9): 772-780.

Chong, J., A. Poutaraud and P. Hugueney (2009). "Metabolism and roles of stilbenes in plants." Plant Science **177**(3): 143-155.

Concibido, V. C., B. W. Diers and P. R. Arelli (2004). "A decade of qtl mapping for cyst nematode resistance in soybean." Crop science **2004 v.44 no.4**(no. 4): pp. 1121-1120.

Cook, D. E., A. M. Bayless, K. Wang, X. Guo, Q. Song, J. Jiang and A. F. Bent (2014). "Distinct copy number, coding sequence, and locus methylation patterns underlie rhg1-mediated soybean resistance to soybean cyst nematode." Plant Physiol **165**(2): 630-647.

Cook, D. E., T. G. Lee, X. Guo, S. Melito, K. Wang, A. M. Bayless, J. Wang, T. J. Hughes, D. K. Willis, T. E. Clemente, B. W. Diers, J. Jiang, M. E. Hudson and A. F. Bent (2012). "Copy number variation of multiple genes at *rhg1* mediates nematode resistance in soybean." Science **338**(6111): 1206-1209.

Council, U. S. G. (2019). "Converting grain units." Retrieved 9/11/2019, from https://grains.org/markets-tools-data/tools/converting-grain-units/.

Epps, J. M. (1971). "Recovery of soybean cyst nematodes (*heterodera glycines*) from the digestive tracts of blackbirds." J Nematol **3**(4): 417-419.

Finer, J. J., P. Vain, M. W. Jones and M. D. McMullen (1992). "Development of the particle inflow gun for DNA delivery to plant cells." Plant Cell Rep **11**(7): 323-328.

Finkle, B. J. and S. H. Kelly (1974). "Catechol o-methyltransferases in pampas grass: Differentiation of m- and p-methylating activities." Phytochemistry **13**(9): 1719-1725.

Gallage, N. J., E. H. Hansen, R. Kannangara, C. E. Olsen, M. S. Motawia, K. Jørgensen, I. Holme, K. Hebelstrup, M. Grisoni and B. L. Møller (2014). "Vanillin formation from ferulic acid in vanilla planifolia is catalysed by a single enzyme." Nature communications **5**: 4037-4037.

Guerinot, M. L., B. A. Morisseau and T. Klapatch (1990). "Electroporation of bradyrhizobium japonicum." Molecular and General Genetics MGG **221**(2): 287-290.

Hahn, M. and H. Hennecke (1984). "Localized mutagenesis in rhizobium japonicum." Molecular and General Genetics MGG **193**(1): 46-52.

Hansen, E. H., B. L. Moller, G. R. Kock, C. M. Bunner, C. Kristensen, O. R. Jensen, F. T. Okkels, C. E. Olsen, M. S. Motawia and J. Hansen (2009). "De novo biosynthesis of vanillin in fission yeast (schizosaccharomyces pombe) and baker's yeast (saccharomyces cerevisiae)." Appl Environ Microbiol **75**(9): 2765-2774.

Hattermann, D. R. and G. Stacey (1990). "Efficient DNA transformation of bradyrhizobium japonicum by electroporation." Appl Environ Microbiol **56**(4): 833-836.

Heeb, S., Y. Itoh, T. Nishijyo, U. Schnider, C. Keel, J. Wade, U. Walsh, F. O'Gara and D. Haas (2000). "Small, stable shuttle vectors based on the minimal pvs1 replicon for use in gram-negative, plant-associated bacteria." Mol Plant Microbe Interact **13**(2): 232-237.

Hernandez, A., J. I. Garcia-Plazaola and J. M. Becerril (1999). "Glyphosate effects on phenolic metabolism of nodulated soybean (glycine max l. Merr.)." Journal of Agricultural and Food Chemistry **47**(7): 2920-2925.

Howland, A., N. Monnig, J. Mathesius, M. Nathan and M. G. Mitchum (2018). "Survey of *heterodera glycines* population densities and virulence pehnotypes during 2015-2016 in missouri." Plant Disease **102**: 2407-2410.

Hu, Y., J. You, C. Li, V. M. Williamson and C. Wang (2017). "Ethylene response pathway modulates attractiveness of plant roots to soybean cyst nematode *heterodera glycines*." Scientific Reports **7**: 41282.

Huettel, R. N. and R. V. Rebois (1986). "Bioassay comparisons for pheromone detection in *heterodera glycines*, the soybean cyst nematode." Proc Helminthological Society of Washington **53**(1): 63-68.

Ibrahim, R. K., V. De Luca, H. Khouri, L. Latchinian, L. Brisson and P. M. Charest (1987). "Enzymology and compartmentation of polymethylated flavonol glucosides in chrysosplenium americanum." Phytochemistry **26**(5): 1237-1245.

Jackson, T. A., G. S. Smith and T. L. Niblack (2005). "*Heterodera glycines* infectivity and egg viability following nonhost crops and during overwintering." Journal of nematology **37**(3): 259-264.

Jaffe, H., R. N. Huettel, A. B. Demilo, D. K. Hayes and R. V. Rebois (1989). "Isolation and identification of a compound from soybean cyst nematode, *heterodera glycines*, with sex pheromone activity." J Chem Ecol **15**(7): 2031-2043.

Johansen, K. T., S. G. Wubshet, N. T. Nyberg and J. W. Jaroszewski (2011). "From retrospective assessment to prospective decisions in natural product isolation: Hplc-spe-nmr analysis of carthamus oxyacantha." Journal of natural products **74**(11): 2454-2461.

Kakkar, S. and B. Souravh (2014). "A review on protocatechuic acid and its pharmacological potential." ISRN Pharmacology **2014**.

Kallscheuer, N., M. Vogt, J. Kappelmann, K. Krumbach, S. Noack, M. Bott and J. Marienhagen (2016). "Identification of the phd gene cluster responsible for phenylpropanoid utilization in corynebacterium glutamicum." Appl Microbiol Biotechnol **100**(4): 1871-1881.

Klink, V. P., K. H. Kim, V. Martins, M. H. Macdonald, H. S. Beard, N. W. Alkharouf, S. K. Lee, S. C. Park and B. F. Matthews (2009). "A correlation between host-mediated expression of parasite genes as tandem inverted repeats and abrogation of development of female *heterodera glycines* cyst formation during infection of *glycine max*." Planta **230**(1): 53-71.

Koenning, S. R. and K. R. Barker (1995). "Soybean photosynthesis and yield as influenced by *heterodera glycines*, soil type and irrigation." Journal of Nematology **27**: 51-62.

Kovach, M. E., P. H. Elzer, D. S. Hill, G. T. Robertson, M. A. Farris, R. M. Roop, 2nd and K. M. Peterson (1995). "Four new derivatives of the broad-host-range cloning vector pbbr1mcs, carrying different antibiotic-resistance cassettes." Gene **166**(1): 175-176.

Kunjapur, A. M. and K. L. J. Prather (2019). "Development of a vanillate biosensor for the vanillin biosynthesis pathway in e. Coli." ACS Synthetic Biology **8**(9): 1958-1967.

Lam, K. C., R. K. Ibrahim, B. Behdad and S. Dayanandan (2007). "Structure, function, and evolution of plant o-methyltransferases." Genome **50**(11): 1001-1013.

Legrand, M., B. Fritig and L. Hirth (1978). "O-diphenol o-methyltransferases of healthy and tobacco-mosaic-virus-infected hypersensitive tobacco." Planta **144**(1): 101-108.

Li, J., T. C. Todd, T. R. Oakley, J. Lee and H. N. Trick (2010). "Host-derived suppression of nematode reproductive and fitness genes decreases fecundity of *heterodera glycines* ichinohe." Planta **232**(3): 775-785.

Lin, J., M. Mazarei, N. Zhao, C. N. Hatcher, W. A. Wuddineh, M. Rudis, T. J. Tschaplinski, V. R. Pantalone, P. R. Arelli, T. Hewezi, F. Chen and C. N. Stewart Jr (2016). "Transgenic soybean overexpressing gmsamt1 exhibits resistance to multiple-hg types of soybean cyst nematode *heterodera glycines*." Plant Biotechnology Journal **14**(11): 2100-2109.

Liu, S., P. K. Kandoth, S. D. Warren, G. Yeckel, R. Heinz, J. Alden, C. Yang, A. Jamai, T. El-Mellouki, P. S. Juvale, J. Hill, T. J. Baum, S. Cianzio, S. A. Whitham, D. Korkin, M. G. Mitchum and K. Meksem (2012). "A soybean cyst nematode resistance gene points to a new mechanism of plant resistance to pathogens." Nature **492**: 256.

Lydon, J. and S. O. Duke (1988). "Glyphosate induction of elevated levels of hydroxybenzoic acids in higher plants." Journal of Agricultural and Food Chemistry **36**(4): 813-818.

Manosalva, P., M. Manohar, S. H. von Reuss, S. Chen, A. Koch, F. Kaplan, A. Choe, R. J. Micikas, X. Wang, K.-H. Kogel, P. W. Sternberg, V. M. Williamson, F. C. Schroeder and

D. F. Klessig (2015). "Conserved nematode signalling molecules elicit plant defenses and pathogen resistance." Nature Communications **6**(1): 7795.

Marinus, M. G., M. Carraway, A. Z. Frey, L. Brown and J. A. Arraj (1983). "Insertion mutations in the dam gene of escherichia coli k-12." Mol Gen Genet **192**(1-2): 288-289.

Masamune, T., M. Anetai, M. Takasugi and N. Katsui (1982). "Isolation of a natural hatching stimulus, glycinoeclepin a, for the soybean cyst nematode." Nature **297**(5866): 495-496.

Mayer, M. J., A. Narbad, A. J. Parr, M. L. Parker, N. J. Walton, F. A. Mellon and A. J. Michael (2001). "Rerouting the plant phenylpropanoid pathway by expression of a novel bacterial enoyl-coa hydratase/lyase enzyme function." The Plant Cell **13**(7): 1669-1682.

Meksem, K., P. Pantazopoulos, V. N. Njiti, L. D. Hyten, P. R. Arelli and D. A. Lightfoot (2001). "'forrest' resistance to the soybean cyst nematode is bigenic: Saturation mapping of the rhg1and rhg4 loci." Theoretical and Applied Genetics **103**(5): 710-717.

Mencherini, T., P. Picerno, M. Festa, P. Russo, A. Capasso and R. Aquino (2011). "Triterpenoid constituents from the roots of paeonia rockii ssp. Rockii." Journal of Natural Products **74**(10): 2116-2121.

Meyer, S. L. and R. N. Huettel (1996). "Application of a sex pheromone, pheromone analogs, and *verticillium lecanii* for management of *heterodera glycines*." J Nematol **28**(1): 36-42.

Niblack, T. L., P. R. Arelli, G. R. Noel, C. H. Opperman, J. H. Orf, D. P. Schmitt, J. G. Shannon and G. L. Tylka (2002). "A revised classification scheme for genetically diverse populations of *heterodera glycines*." Journal of Nematology **34**(4): 279-288.

Nonaka, S., T. Katsuyama, T. Kondo, Y. Sasaki, T. Asami, S. Yajima and S. Ito (2016). "1,10-phenanthroline and its derivatives are novel hatching stimulants for soybean cyst nematodes." Bioorganic & Medicinal Chemistry Letters **26**(21): 5240-5243.

Okai, N., T. Miyoshi, Y. Takeshima, H. Kuwahara, C. Ogino and A. Kondo (2016). "Production of protocatechuic acid by *corynebacterium glutamicum* expressing chorismate-pyruvate lyase from *escherichia coli*." Applied Microbiology and Biotechnology **100**(1): 135-145.

Patterson, D. T. (1981). "Effects of allelopathic chemicals on growth and physiological responses of soybean (*glycine max*)." Weed Science **29**(1): 53-59.

Pincon, G., S. Maury, L. Hoffmann, P. Geoffroy, C. Lapierre, B. Pollet and M. Legrand (2001). "Repression of o-methyltransferase genes in transgenic tobacco affects lignin synthesis and plant growth." Phytochemistry **57**(7): 1167-1176.

Poromarto, S. H., G. G. Gramig, B. D. Nelson and S. Jain (2015). "Evaluation of weed species from the northern great plains as hosts of soybean cyst nematode." Plant Health Progress **16**(1): 23-28.

Rakwal, R., M. Hasegawa and O. Kodama (1996). "A methyltransferase for synthesis of the flavanone phytoalexin sakuranetin in rice leaves." Biochemical and Biophysical Research Communications **222**(3): 732-735.

Ramachandra Rao, S. and G. A. Ravishankar (2000). "Vanilla flavour: Production by conventional and biotechnological routes." Journal of the Science of Food and Agriculture **80**(3): 289-304.

Reigosa, M. J. and E. Malvido-Pazos (2007). "Phytotoxic effects of 21 plant secondary metabolites on arabidopsis thaliana germination and root growth." J Chem Ecol **33**(7): 1456-1466.

Rende, J. F., P. M. Tefft and L. W. Bone (1982). "Pheromone attraction in the soybean cyst nematode *heterodera glycines* race 3." Journal of Chemical Ecology **8**(6): 981-991.

Riggs, R. D. (1987). "Nonhost root penetration by soybean cyst nematode." Journal of nematology **19**(2): 251-254.

Riggs, R. D. (1992). Host range. Biology and management of the soybean cyst nematode, American Phytopathological Society
107-114.

Riggs, R. D. and D. P. Schmitt (1988). "Complete characterization of the race scheme for *heterodera glycines*." Journal of nematology **20**(3): 392-395.

S Freitas, C., G. Alves da Silva, D. Perrone, M. A Vericimo, D. Dos S Baião, P. R Pereira, V. M F Paschoalin and E. M Del Aguila (2018). "Recovery of antimicrobials and bioaccessible isoflavones and phenolics from soybean (*glycine max*) meal by aqueous extraction." Molecules (Basel, Switzerland) **24**(1): 74.

Shilling, D. G. and F. Yoshikawa (1987). A rapid seedling bioassay for the study of allelopathy. Allelochemicals: Role in agriculture and forestry, American Chemical Society. **330:** 334-342.

Shimizu, M., Y. Kobayashi, H. Tanaka and H. Wariishi (2005). "Transportation mechanism for vanillin uptake through fungal plasma membrane." Appl Microbiol Biotechnol **68**(5): 673-679.

Stalikas, C. D. (2007). "Extraction, separation, and detection methods for phenolic acids and flavonoids." Journal of Separation Science **30**(18): 3268-3295.

Stuurman, N., C. Pacios Bras, H. R. Schlaman, A. H. Wijfjes, G. Bloemberg and H. P. Spaink (2000). "Use of green fluorescent protein color variants expressed on stable broad-host-range vectors to visualize rhizobia interacting with plants." Mol Plant Microbe Interact **13**(11): 1163-1169.

Subbotin, S. A., M. Mundo-Ocampo and J. G. Baldwin (2010). Systematics of the heteroderinae. Systematics of cyst nematodes (nematoa: Heteroderinae), Koninklijke Brill NV**:** 101-106.

Tefft, P. M. and L. W. Bone (1985). "Plant-induced hatching of eggs of the soybean cyst nematode *heterodera glycines*." J Nematol **17**(3): 275-279.

Trick, H., R. D. Dinkins, E. R. Santarem, R. Di, V. Samoylov, C. A. Meurer, D. R. Walker, W. A. Parrott, J. J. Finer and G. B. Collins (1997). "Recent advances in soybean transformation." Plant Tissue Culture and Biotechnology **3**(9-26).

USDA, N. A. S. S. "Quick stats; 2014 soybean price recieved $ / bu " Retrieved 9/11/19, from https://quickstats.nass.usda.gov/results/4FCC4551-4AE3-35D5-9064-9095DF71415F.

Vincze, E. and S. Bowra (2006). "Transformation of rhizobia with broad-host-range plasmids by using a freeze-thaw method." Appl Environ Microbiol **72**(3): 2290-2293.

Wang, T. S. C., T. Yang and T. Chuang (1967). "Soil phenolic acids as plant growth inhibitors." Soil Science **103**(4): 239-246.

Wen, L., C. Yuan, T. K. Herman and G. L. Hartman (2017). "Accessions of perennial *glycine* species with resistance to multiple types of soybean cyst nematode (*heterodera glycines*)." Plant Disease **101**(7): 1201-1206.

Winstead, N. N., C. B. Skotland and J. N. Sasser (1995). "Soybean cyst nematode in north carolina " Plant Disease Reporter **39**: 9-11.

Wrather, J. A. and S. R. Koenning (2006). "Estimates of disease effects on soybean yields in the united states 2003 to 2005." J Nematol **38**(2): 173-180.

Xing, L. and A. Westphal (2013). "Synergism in the interaction of *fusarium virguliforme* with *heterodera glycines* in sudden death syndrome of soybean." Journal of Plant Diseases and Protection **120**(5): 209-217.

Yang, J.-H., T. P. Kondratyuk, K. C. Jermihov, L. E. Marler, X. Qiu, Y. Choi, H. Cao, R. Yu, M. Sturdy, R. Huang, Y. Liu, L.-Q. Wang, A. D. Mesecar, R. B. van Breemen, J. M. Pezzuto, H. H. S. Fong, Y.-G. Chen and H.-J. Zhang (2011). "Bioactive compounds from the fern lepisorus contortus." Journal of natural products **74**(2): 129-136.

Young, L. D. (1992). Epiphytology and life cycle. Biology and management of the soybean cyst nematode. R. D. Riggs and A. Wrather**:** 27-36.

Zenk, M. H. (1965). "Biosynthese von vanillin in *vanilla planifolia* andr." Zeitschrift für Pflanzenphysiologie **53**.

Chapter 4 - General Materials and Methods

Software for statistical analysis and graphics

Statistical analysis and graphs were done in R-Studio version 1.0.153, running R version 3.6.1 for 64-bit Microsoft Windows (R Development Core Team 2013). Analysis of variance was done with the "anova" function with data being fit to a linear model (Chambers, Hastie et al. 1990). Dunnett's tests were done with the general linear hypothesis test ("glht"), function which is part of the Multicomp package developed by Torsten Hothorn (2008). Dunn's test of multiple comparisons using rank sums was performed with the dunn.test package and function developed by Alex Dinno using the Bonferroni adjustment method. Kruskal-Wallis rank sum, Pearson's Chi-square, and Tukey's honest significant difference tests were performed with the "kruskal.test", "chisq.test", and "TukeyHSD" functions, respectively, which are part of the stats package for R.

Boxplots were generated using the "boxplot" function in R, which is part of the Bioconductor BiocGenerics package version 0.30.0 (Huber, Carey et al. 2015). Whiskers on box-plots represent the minimum of either 1.5 times the interquartile range or the extreme of the sample range. To generate AA sequence alignment graphics, sequences were first loaded into ClustalX version 2.1 and aligned with a Gonnet series protein weight matrix (Thompson, Gibson et al. 1997). Clustal alignments were then loaded into Jalview version 2.11.0 and graphics generated using a custom color scheme based on the PAM250 weight matrix and disulfide bridge formation importance (Waterhouse, Procter et al. 2009). Photos were edited with Adobe Photoshop version 7.0 for brightness and contrast to convey information, resizing, cropping, and to add text/figures. Figure 2.7 is a stacked image for greater depth-of-field.

Transgene optimization

Native nucleic acid sequences of genes to be used in this work were modified for optimized expression in plants. Because the native nucleic acid sequence of the antimicrobial peptide (AMP) juruin is not known, the online EMBOSS backtranslation tool, backtranseq was used to derive its sequence using *Zea mays* most likely used codons (Rice, Longden et al. 2000). AA sequences used for VA work were reverse translated using bioinformatics.org's reverse translate tool using *G. max*'s most commonly used codons (Stothard 2000). Codon optimization was modeled after work done by Jackson et al. (2014) in which RNA instability motifs from Table 4.1 were removed. This paper showed that transgene optimization in this way increased expression 5- to 60-fold in plants. For AMP genes the extremely rare monocot codon TTA was changed to either the most commonly used leucine codon, CTC, or another synonymous codon not offending subsequent requirements. Synonymous codons were used to avoid restriction sites used in molecular subcloning to prevent digestion of protein coding sequences (CDSs) during this process. This included *Bam*HI and *Not*I for AMP genes and *Bam*HI, *Hind*III, and *Not*I for VA genes. Codon optimization was complete after iterations of removing instability motifs, low use codons (for AMP genes), and restriction sites resulted in the exclusion of these elements. AMP's were synthesized in tandem, each with its own 5' angiosperm context sequence (CACAA) and 5' & 3' restriction sites for subcloning. Differences in optimization strategies can be seen from the optimized zeamatin CDS done by this author and Genscript in Appendix A. Optimized genes were synthesized and cloned into the pUC57 cloning vector via *Eco*RV blunt-ended digestion and re-ligation by Genscript (Piscataway, NJ). The versatility of the resulting plasmid through subcloning and adapter ligation can be seen in Figure 4.1. Due to their similar

size, 3-dehydroshikimate dehydratase (3DSD) and catechol o-methyltransferase (COMT) CDSs used in this research were synthesized and cloned into separate plasmids by Genscript.

Instability element	Sequence
Cryptic splice donor	MAGGTRAGT
Cryptic splice acceptor	YYYYNTAGG
RNA destabilizing sequence	ATTTA
RNA instability determinant	ATAGAT
NUE1	ATAAA
NUE2	WWWWWW
FUE-TRE-CE	TTTT
FUE-ORY1	WWWWGT
FUE-ORY2	GTGTG
FUE-ORY3	TGTAW
FUE-ORY4	TGTGT
FUE-ORY5	WTGTA
ATRICH1	WSWWWW
ATRICH2	WWSWWW
ATRICH3	WWWSWW
ATRICH4	WWWWSW

Table 4.1 Nucleic acid sequences removed from transgenes for increased mRNA stability

IUPAC code used for ambiguous bases

General subcloning procedures

Genetic material to be manipulated for subcloning was first harvested from *E. coli* strain DH5α by using culture from a fresh streak plate to inoculate the appropriate amount of liquid LB media for mini/midi/maxi-prep containing selective antibiotics and incubating overnight (~14-16 hr) on an orbital shaker at 200 rpm, 37°C. Isolates were previously confirmed to harbor the

correct plasmid via colony PCR. Plasmids were harvested from bacteria with an E.Z.N.A mini/midi/maxi-prep kit from Omega Bio-tek (Norcross, GA) following manufacturer recommendations. The concentration of plasmid solutions was determined by 260 nm absorbance on a Nanodrop ND-1000 spectrophotometer (ThermoFisher). For subcloning with PCR products, Platinum® *Taq* high fidelity DNA polymerase from Invitrogen (Irvine, CA) was used for amplicon production following manufacturer recommendations followed by PCR clean up by running amplicons on a 0.8% agarose gel at 100V for a minimum of 20 min and extracting the amplicon from the gel as described below.

Restriction digestion for a typical subcloning reaction used 1 µg of insert plasmid and 2.5 µg of vector plasmid. Restriction digestions were carried out following manufacture recommendations (Promega: San Luis Obispo, CA; New England BioLabs: Ipswich, MA; Takara Bio: Mountain View, CA) with the vector reactions receiving 1 µL of Promega 1 unit μL^{-1} thermosensitive shrimp alkaline phosphatase (tSAP) to prevent self-ligation. Restriction digestions were carried out in a water bath at the appropriate temperature for a minimum of 1 hr. After digestion, restriction enzymes were inactivated by incubating at 65°C for 15 min. (74°C for 15 min for tSAP reactions) in a water bath. Reactions were run against undigested plasmids on a 0.8% agarose gel for a minimum of 20 min to resolve fragments. DNA fragments of interest were excised from the gel with a sterile razor blade and purified with a Zymo Research (Irvine, CA) gel DNA recovery kit following manufacturer recommendations. DNA concentrations of the resulting solutions were measured via 260 nm absorbance and used for ligation reactions.

Ligation reactions were performed with Promega T4 DNA ligase following manufacturer recommendations with an insert-to-vector molar ratio of 3:1. The insert-to-vector ratio was modified for recalcitrant ligation reactions typically in favor of greater

insert. Ligation reactions were incubated for 3 hr at RT or overnight (~14-16 hr) at 4°C. After incubation, 1-3 µL of ligation reaction was used for transformation of homemade chemically competent DH5α E. coli. Transformants were confirmed to harbor engineered plasmids via colony PCR with GOI and vector specific PCR primers. Sequence confirmation of engineered plasmids was done with Sanger sequencing through the Kansas State University Sequencing Facility, Agronomy (no longer operational) or Genewiz (South Plainfield, NJ).

Bacterial transformation procedures

Chemically competent DH5α *E. coli* cells were produced for plasmid amplification and subcloning procedures. This protocol was developed from one used by the Krantz lab of University of California Berkeley (Krantz). For this, 500 mL liquid LB media was inoculated with 1-3 mL overnight culture of W/T cells and incubated at 37°C on an orbital shaker until an OD_{600} of 0.4 was reached. The culture was transferred to pre-chilled 250 mL centrifuge bottles and incubated on ice for 20-30 min. Cultures were then centrifuged in a swing-bucket rotor at 3 k·*g* for 15 min at 4°C. The resulting supernatant was decanted and each pellet was resuspended with 100 mL ice cold 100 mM $MgCl_2$ solution. Solutions were combined into one 250 mL centrifuge bottle and then centrifuged at 2 k·*g* for 15 min at 4°C. The resulting supernatant was decanted and each pellet was resuspended with 100 mL ice cold 100 mM $CaCl_2$. Samples were then incubated on ice for 20 min and then centrifuged at 2 k·*g* for 15 min at 4°C. Supernatant were decanted and each pellet was resuspended with 25 mL ice cold 85 mM $CaCl_2$, 15% glycerol solution. The resulting solution was then centrifuged at 1 k·*g* for 15 min at 4°C. The supernatant was decanted and the pellets were resuspended in 1 mL ice cold 85 mM $CaCl_2$, 15% glycerol

solution. Fifty microliters of the resulting bacterial solution was aliquoted into 1.5 mL centrifuge tubes and frozen with liquid nitrogen. Competent cells were stored in a -80°C freezer until used.

Competent *E. coli* cells were transformed similarly to that of any commercially available chemically competent *E. coli* cells. Briefly, 1.5 mL centrifuge tubes with competent cells were thawed on ice. Afterwards, 1-3 µL of 50 ng μL^{-1} plasmid solution or ligation reaction was added and mixed by gently tapping with the forefinger. Microfuge tubes were incubated on ice for 10-30 min. Reactions were heat shocked at 42°C for 30 sec followed by a 3 min incubation on ice. After which, 250 µL of antibiotic-free LB broth was added and samples incubated for 1 hr at 37°C on an orbital shaker, at 200 rpm. After incubation, 60-100 µL of transformation reaction was plated on LB plates with the appropriate antibiotics for selection of transformants. Plates were incubated overnight at 37°C and transformation was confirmed with colony PCR using the appropriate primers from Appendix C. Competent *Agrobacterium tumefaciens* in this research were made and transformed according to a protocol developed by Höfgen and Willmitzer (1988) with LB media substituting YEB media.

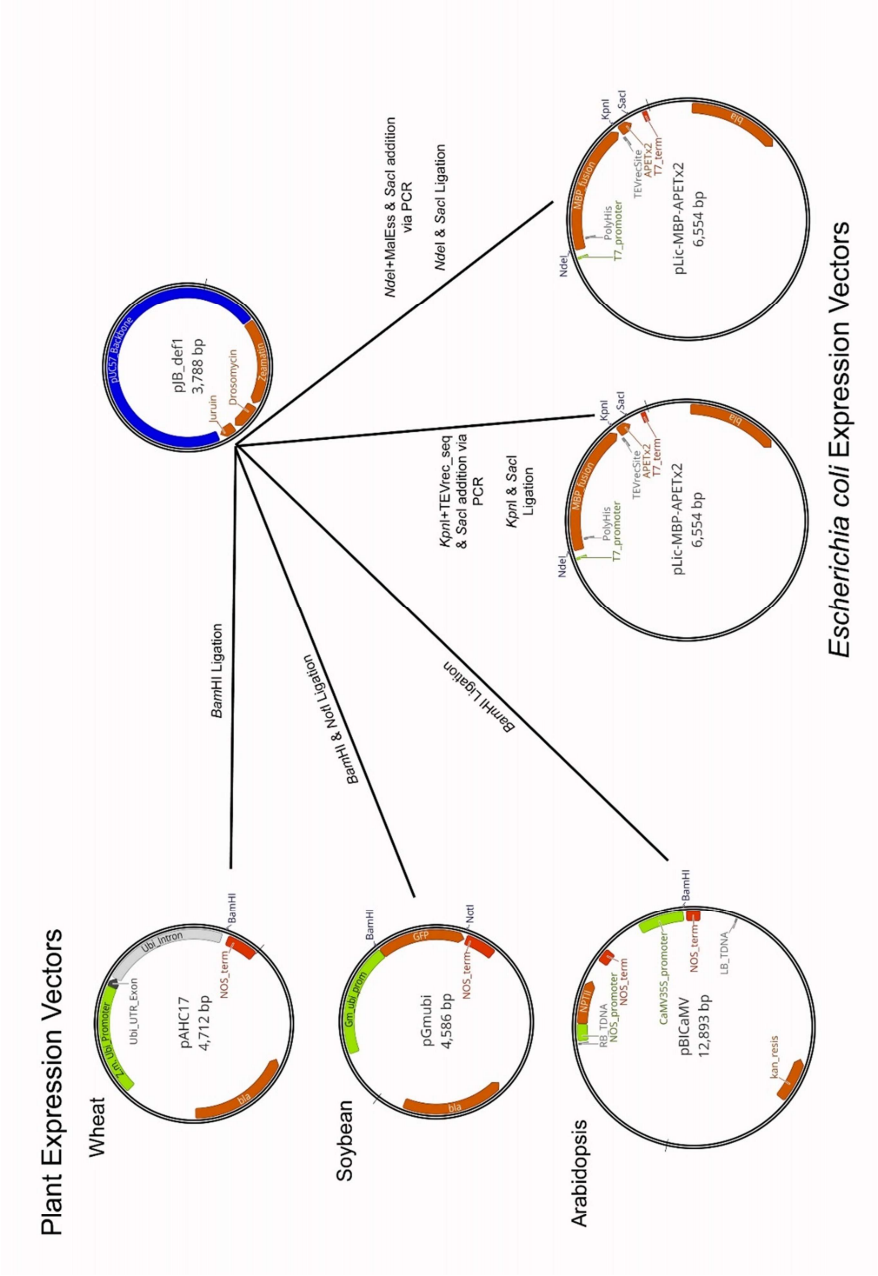


Figure 4.1 Recombinant AMP subcloning into various expression vectors

Recombinant AMP CDSs juruin, drosomycin, and zeamatin were independently subcloned using restriction digestion and ligation into plant expression vectors optimized for monocots (pAHC17), dicots (pGmubi), or general overexpression for both via *Agrobacterium*-mediated transformation (pBICaMV). Genetic elements such as alternate restriction sites and or cleavage sites were added/removed via PCR primer modification to construct bacterial expression vectors (pLic-MBP-APETx2 backbone) that generate recombinant proteins for different applications.

DNA isolation from plants

For isolating genomic DNA from plants, a Tris-EDTA-SDS method was used. For this protocol about 300 mg leaf tissue was collected in 2.0 microfuge tube containing a metal bead and was immediately flash frozen in liquid nitrogen. Tissue was pulverized with a Qiagen TissueLyser II. 500 µL extraction buffer (200 mM Tris pH 7.5, 250 mM NaCl, 25mM EDTA pH 8.0, and 0.5% SDS) was added to each microfuge tube along with 5 ng RNase A (Omega Bio-tek). Samples were vortexed until homogenized, after which, the metal bead was removed. The microfuge tubes were incubated at 65 °C for 5-10 min for digestion of RNA followed by centrifugation at 14 k·*g* for two minutes. The supernatant was moved to a new microfuge tube. An equal volume isopropanol was added to each sample and mixed by inverting the microfuge tube. Samples were left at room temperature to precipitate DNA for two min. Samples were centrifuged at 14 k·*g* for two minutes, after which the supernatant was poured off. The remaining DNA pellet was washed with 1 mL 70% ethanol. Samples were centrifuged at 14 k·*g* for one minute and ethanol poured off. Excess ethanol was removed by pipette and remaining was evaporated by leaving microfuge tubes open. The resulting pellet was resuspended in 50-100 µL of elution buffer (10 mM Tris-HCl 8.0). Samples were centrifuged at 14 k·*g* for one minute to pellet insoluble debris and the resulting supernatant containing genomic DNA was transferred to a new microfuge tube. DNA for tan spot bioassay two was isolated with automated equipment in the Guttieri lab, USDA-ARS Manhattan, KS. For this leaf tissue was collected in a CRD fashion into 96-well plates. Plates were loaded into an oKtopure automated DNA extraction system (Biosearch Technologies: Middlesex, UK) for DNA isolation. PCR reactions for these samples were performed in 96-well plates with PCR master mixes being dispensed by a Mantis® liquid handler (Formulatrix®: Bedford, MA) in a CRD fashion.

Bacterial strains

pUC57

Transgenes were synthesized into the bacterial cloning vector pUC57 by Genscript. pUC57 confers ampicillin resistance via beta-lactamase. High copy number is maintained in *E. coli* via pMB1 ori. A multiple cloning site (MCS) within a lacZ gene facilitates subcloning. An *E. coli* expression vector was generated from the pGa-COMT Genscript vector via *Bam*HI digestion and self-ligation. The resulting plasmid, pUC57m, is a ΔlacZ60-63GAT mutant. In-frame cloning into one of eight unique restriction sites will give a 5' *lacZ* fusion driven by the *lac* promoter.

DH5α

E. coli used for molecular subcloning is strain DH5α developed by Douglas Hanahan (1983) and is also known by the commercial name of TOP10. This strain is non-pathogenic and derived from the K12 isolate and subsequently DH1. DH5α has a high transformation efficiency and yields large amounts of high fidelity, methylated plasmids due to reduced endonuclease and homologous recombination activities compared to other strains of *E. coli*. *Dam* and *Dcm* activity in this strain methylates plasmids for protection against restriction digestion. The genotype for DH5α is *supE44*, *ΔlacU169* (φ80 *lacZΔM15*) *hsdR17 recA1 endA1 gyrA96 thi-1 relA1* (Brown 1991).

BL21 (DE3)

E. coli used for recombinant protein production in this work is strain BL21 (DE3). This strain has a K12 background and was developed by Studier and Moffatt (1986). In this isolate, expression of transgenes downstream of a T7 promoter is driven by the highly selective bacteriophage T7 RNA polymerase. This polymerase was integrated into the genome of this strain on lambda phage DE3. This strain of *E. coli* works well for production of recombinant proteins due it reduced protease activity (Grodberg and Dunn 1988). The genotype for BL21 (DE3) is *hsdS gal* (λ*c*Its857 *ind*1 *S*am7 *nin*5 *lacUV5*-T7 gene 1) (Brown 1991).

EHA105

The *Agrobacterium tumefaciens* strain EHA105 used for *A. thaliana* transformation in this work has a C58 chromosomal background made up of a linear and circular chromosome, and two circular plasmids. Virulence factors are found on all four of these genetic elements (Goodner, Hinkle et al. 2001). EHA105 is a derivative of EHA101. The disarmed Ti helper plasmid of EHA105, pEHA105, was constructed by and named after Elizabeth Hood (1993). pEHA105 evolved from the replacement of the T-DNA region of pTiBo542, a hypervirulent Ti plasmid from *A. tumefaciens* strain A281, with a kanamycin-resistance gene. This kanamycin-resistance gene was then removed via double cross-over with the intermediate plasmid pπδ313. The pπδ313 intermediate plasmid was then removed to increase versatility of the strain for its use in biotechnology by limiting plasmid incompatibility groups within a single bacteria (Hood, Helmer et al. 1986, Hood, Gelvin et al. 1993). Floral dip transformation of *A. thaliana* in this work was performed as described by Clough and Bent (1998). The binary vector used for this

transformation was pBICaMV with CDSs subcloned via *Bam*HI (AMP CDSs) or *Hind*III (VA CDSs) restriction digestion and ligation Figure 4.2.

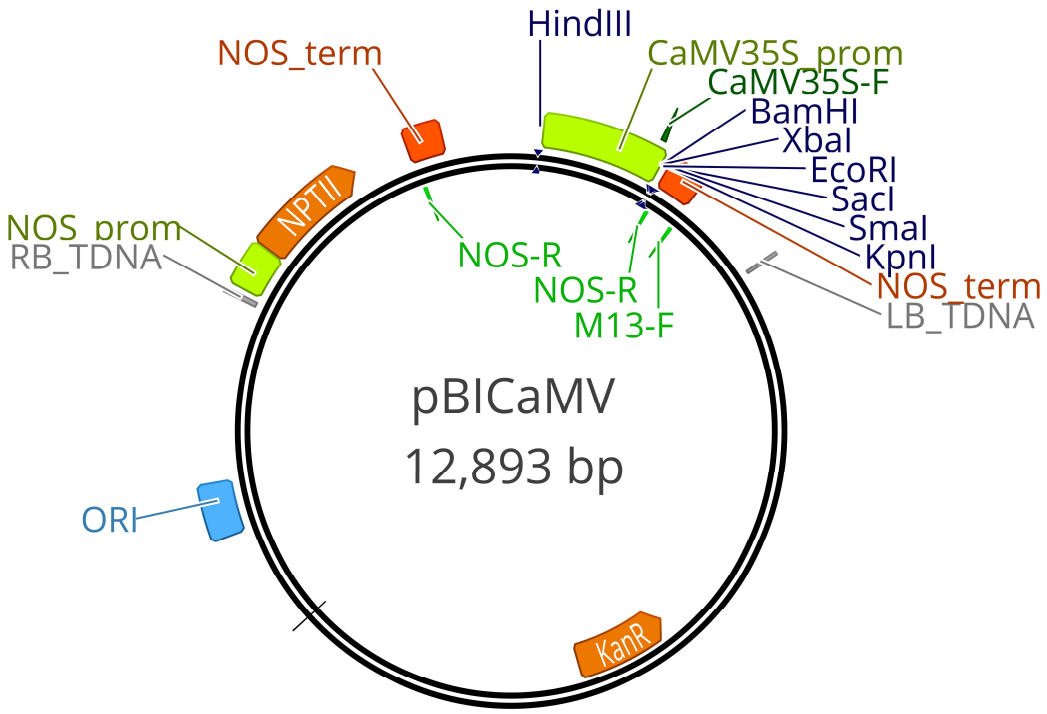


Figure 4.2 Diagram of pBICaMV plasmid

Abbreviations include the regulatory elements origin of replication (ORI), right and left border transfer DNA (RB_TDNA, LB_TDNA), nopaline synthase promoter (NOS_prom), neomycin phosphotransferase II (*NPTII*) gene for kanamycin selection in plants, a kanamycin resistance gene (*KanR*) for selection in bacteria, nopaline synthase terminators (NOS_term), cauliflower mosaic virus 35s promoter (CaMV35S_prom), forward (*-F) and reverse (*.R) primer binding sites, and unique restriction sites.

Works Cited

Brown, T. A. (1991). Molecular biology labfax, BIOS Scientific Publishers Limited

Chambers, J., T. Hastie and D. Pregibon (1990). Statistical models in s. Compstat, Heidelberg, Physica-Verlag HD.

Clough, S. J. and A. F. Bent (1998). "Floral dip: A simplified method for agrobacterium-mediated transformation of arabidopsis thaliana." Plant J **16**(6): 735-743.

Goodner, B., G. Hinkle, S. Gattung, N. Miller, M. Blanchard, B. Qurollo, B. S. Goldman, Y. Cao, M. Askenazi, C. Halling, L. Mullin, K. Houmiel, J. Gordon, M. Vaudin, O. Iartchouk, A. Epp, F. Liu, C. Wollam, M. Allinger, D. Doughty, C. Scott, C. Lappas, B. Markelz, C. Flanagan, C. Crowell, J. Gurson, C. Lomo, C. Sear, G. Strub, C. Cielo and S. Slater (2001). "Genome sequence of the plant pathogen and biotechnology agent agrobacterium tumefaciens c58." Science **294**(5550): 2323-2328.

Grodberg, J. and J. J. Dunn (1988). "Ompt encodes the escherichia coli outer membrane protease that cleaves t7 rna polymerase during purification." J Bacteriol **170**(3): 1245-1253.

Hanahan, D. (1983). "Studies on transformation of escherichia coli with plasmids." Journal of Molecular Biology **166**(4): 557-580.

Höfgen, R. and L. Willmitzer (1988). "Storage of competent cells for agrobacterium transformation." Nucleic Acids Research **16**(20): 9877.

Hood, E., S. Gelvin, L. S. Melchers and A. Hoekema (1993). "New agrobacterium helper plasmids for gene transfer to plants." Transgenic Research **2**: 208-218.

Hood, E. E., G. L. Helmer, R. T. Fraley and M. D. Chilton (1986). "The hypervirulence of agrobacterium tumefaciens a281 is encoded in a region of ptibo542 outside of t-DNA." Journal of Bacteriology **168**(3): 1291.

Hothorn, T., F. Bretz and P. Westfall (2008). "Simultaneous inference in general parametric models." Biometrical Journal **50**(3): 346-363.

Huber, W., V. J. Carey, R. Gentleman, S. Anders, M. Carlson, B. S. Carvalho, H. C. Bravo, S. Davis, L. Gatto, T. Girke, R. Gottardo, F. Hahne, K. D. Hansen, R. A. Irizarry, M. Lawrence, M. I. Love, J. MacDonald, V. Obenchain, A. K. Oleś, H. Pagès, A. Reyes, P. Shannon, G. K. Smyth, D. Tenenbaum, L. Waldron and M. Morgan (2015). "Orchestrating high-throughput genomic analysis with bioconductor." Nature Methods **12**(2): 115-121.

Jackson, M. A., P. R. Sternes, S. R. Mudge, M. W. Graham and R. G. Birch (2014). "Design rules for efficient transgene expression in plants." Plant Biotechnol J **12**(7): 925-933.

Krantz, B. "Making calcium competent cells." 2017, from http://mcb.berkeley.edu/labs/krantz/protocols/calcium_comp_cells.pdf.

R Development Core Team (2013). R: A language and environment for statistical computing. Vienna, Austria, R Foundation for Statistical Computing

Rice, P., I. Longden and A. Bleasby (2000). "Emboss: The european molecular biology open software suite." Trends in genetics : TIG **16**(6): 276-277.

Stothard, P. (2000). "The sequence manipulation suite: Javascript programs for analyzing and formatting protein and DNA sequences." Biotechniques **28**(6): 1102, 1104.

Studier, F. W. and B. A. Moffatt (1986). "Use of bacteriophage t7 rna polymerase to direct selective high-level expression of cloned genes." Journal of Molecular Biology **189**(1): 113-130.

Thompson, J. D., T. J. Gibson, F. Plewniak, F. Jeanmougin and D. G. Higgins (1997). "The clustal_x windows interface: Flexible strategies for multiple sequence alignment aided by quality analysis tools." Nucleic Acids Res **25**(24): 4876-4882.

Waterhouse, A. M., J. B. Procter, D. M. Martin, M. Clamp and G. J. Barton (2009). "Jalview version 2--a multiple sequence alignment editor and analysis workbench." Bioinformatics **25**(9): 1189-1191.

Appendix A - Nucleic and Amino Acid Sequences of Interest for Antifungal Work

Lowercase bolded text differs from original sequence; uppercase bolded text with underline show start and stop codons; grayed text shows documented or SignalP-5.0 predicted export signal sequences

>gi|162458912|ref|NM_001111886.1| Zea mays stress-induced protein1 (sip1), mRNA
GAATTCCGGCTTCAAATACTTGCATAAG**ATG**GCAGGTTCCGTGGCAATCGTGGGCAT
CTTCGTCGCCCTCCTCGCCGTGGCCGGCGAGGCGGCTGTGTTCACGGTGGTGAACCA
GTGCCCGTTCACCGTGTGGGCCGCGTCCGTGCCGGTGGGCGGCGGGCGGCAGCTGA
ACCGCGGCGAGAGCTGGCGGATCACGGCCCCCGCGGGCACGACGGCCGCGCGCATC
TGGGCGCGCACGGGGTGCAAGTTCGACGCGAGCGGGCGCGGGAGCTGCCGCACGG
GGGACTGCGGCGGCGTGCTGCAGTGCACTGGGTACGGGCGCGCGCCCAACACGCTG
GCGGAGTACGCGCTGAAGCAGTTCAACAACCTCGACTTCTTCGACATCTCCCTCATC
GACGGCTTCAACGTGCCCATGAGCTTCCTCCCCGACGGCGGGTCCGGGTGCAGCCGC
GGCCCGCGCTGCGCCGTGGACGTGAACGCGCGCTGCCCTGCCGAGCTGCGGCAGGA
CGGCGTGTGCAACAACGCGTGCCCCGTGTTCAAGAAGGACGAGTACTGCTGCGTCG
GCTCGGCGGCCAACGACTGCCACCCGACCAACTACTCCAGGTACTTCAAGGGGCAG
TGCCCCGACGCGTACAGCTACCCCAAGGATGACGCCACCAGCACCTTCACCTGCCCC
GCCGGAACCAACTACAAGGTCGTCTTCTGCCCG**TGA**GGCCGCTGAACTAGCATCAG
CGTGCGCGCGTCGACCAAGAACAAGAAATAAACGACCGAGGCTGTGTATGTTGCCC
GCCGTGTGCTCAACTGGAGCAAAAATAAAGCCAACAAAACAAGCACGTGTGATCTC
TATGTCACCAACTCTATCACCTTAATTAATGGGGCATTATGAAGTTGTTTCTTTACAT
GCTCGTACTGAAATTGGGTTTTAAAAAAAAAAAAAAAAAAAAAAACCGGAATTC

>Codon optimized zeamatin
ATGGCAGGTTCCGTGGCAATCGTGGGCATCTTCGTCGCCCTCCTCGCCGTGGCCGGC
GAGGC**t**GCTGT**c**TTCACGGTGGTGAACCAGTGCCCGTTCACCGT**t**TGGGCCGCGTCCG
TGCCGGTGGGCGGCGGGCGGCAGCTGAACCGCGGCGAGAGCTGGCGGATCACGGCC
CCCGCGGGCACGACGGCCGCGCGCATCTGGGCGCGCACGGGGTGCAAGTTCGACGC
GAGCGGGCGCGGGAGCTGCCGCACGGGGGACTGCGGCGGCGTGCTGCAGTGCACTG
GGTACGGG**a**G**a**GCGCCCAACACGCTGGCGGAGTACGCGCTGAAGCAGTTCAACAAC
CTCGACTTCTTCGACATCTCCCTCATCGACGGCTTCAACGTGCCCATGAGCTTCCTCC
CCGACGGCGGGTCCGGGTGCAGCCGCGGCCCGCGCTGCGCCGTGGACGTGAACGCG
CGCTGCCCTGCCGAGCTGCGGCAGGACGGCGT**t**TGCAACAACGCGTGCCCCGTGTTC
AAGAAGGACGAGTACTGCTGCGTCGGCTCGGCGGCCAACGACTGCCACCCGACCAA
CTACTCCAGGTACTTCAAGGGGCAGTGCCCCGACGCGTACAGCTACCCCAAGGATG
ACGCCACCAGCACCTTCACCTGCCCCGCCGGAACCAACTACAAGGTCGTCTTCTGCC
CGTGA

>Genscript optimized zeamatin

ATGGC**t**GG**g**TC**g**GT**t**GC**c**ATCGTGGGCATCTTCGTCGC**t**CTCCT**g**GC**t**GT**t**GC**t**GGCGAGG
C**t**GCTGT**t**TTCACGGTGGT**c**AACCA**a**TGCCC**a**TTCACCGT**c**TGGGC**t**GC**t**TCCGT**c**CC**t**GT**t**
GGCGG**g**GG**c**CGGCAGCT**c**AAC**a**G**a**G**g**GGAGAGCTGG**a**G**a**ATCACGGC**t**CC**a**GC**t**GGCAC
cACGGC**t**GC**ta**G**g**ATCTGGGC**ta**G**a**AC**c**GGGTGCAAGTTCGACGC**ttcg**GGG**a**G**a**GG**ctca**TG
C**a**G**a**AC**c**GG**c**GA**t**TGCGG**g**GGCGTGCTGCA**a**TGCAC**g**GGGTACGG**ca**G**g**GC**t**CC**t**AACAC**c**
CT**c**GC**c**GAGTACGCGCTGAAGCAGTTCAACAACCTCGACTTCTTCGA**t**ATC**ag**CCT**g**AT
CGACGGCTTCAACGT**c**CC**a**ATG**tcg**TTCCTCCC**g**GA**t**GG**g**GG**c**TCCGGGTGCAGC**a**G**g**GG
CCC**ta**G**a**TGCGC**t**GTGGACGT**c**AACGC**ta**G**g**TGCCCTGC**t**GAGCTG**a**G**a**CA**a**GACGGCGTG
TGCAACAACGC**c**TGCCCCGT**c**TTCAAGAAGGACGAGTACTGCTGCGT**g**GG**g**TC**a**GC**c**G
C**g**AACGA**t**TGCCACCC**t**ACCAACTACTC**t**AGGTACTTCAAGGG**c**CAGTGCCCCGACGC**t**
TAC**tct**TACCC**t**AAGGA**c**GA**t**GC**t**ACC**tc**CAC**g**TTCACCTGCCC**a**GC**t**GG**c**ACCAACTACAA
GGT**t**GT**g**TTCTGCCCGTGA

>ZeamatinTranslation
MAGSVAIVGIFVALLAVAGEAAVFTVVNQCPFTVWAASVPVGGGRQLNRGESWRITAP
AGTTAARIWARTGCKFDASGRGSCRTGDCGGVLQCTGYGRAPNTLAEYALKQFNNLD
FFDISLIDGFNVPMSFLPDGGSGCSRGPRCAVDVNARCPAELRQDGVCNNACPVFKKDE
YCCVGSAANDCHPTNYSRYFKGQCPDAYSYPKDDATSTFTCPAGTNYKVVFCP

>gi|442629970|ref|NM_079177.4| Drosophila melanogaster drosomycin (Drs), mRNA
CCACAAGTCGCTGATAATTCAAACAGAAATCATTTACCAAGCTCCGTGAGAACCTTT
TCCAAT**ATG**ATGCAGATCAAGTACTTGTTCGCCCTCTTCGCTGTCCTGATGCTGGTG
GTCCTGGGAGCCAACGAGGCCGATGCCGACTGCCTGTCCGGAAGATACAAGGGTCC
CTGTGCCGTCTGGGACAACGAGACCTGTCGTCGTGTGTGCAAGGAGGAGGGACGCT
CCAGTGGCCACTGCAGCCCCAGTCTGAAGTGCTGGTGCGAAGGATGC**TAA**ATCCAT
GAGCAATTAGCATGAACGTTCTGAAAAGCGCGTTTAGCTCTCCACTACTTACACATA
TTCTATGCTGCAATATTGAAAATCTAATAAACAAAACTAATGTACATT

>Codon optimized drosomycin
ATGATGCAGATCAAGTACTTGTTCGCCCTCTTCGCTGTCCTGATGCTGGTGGTCCTGG
GAGCCAACGAGGCCGATGCCGACTGCCTGTCCGGAAG**g**TA**t**AAGGGTCCCTGTGCCG
TCTGGGACAACGAGACCTGTCGTCGTGT**t**TGCAAGGAGGAGGGACGCTCC**tc**TGGCCA
CTGCAGCCCCAGTCTGAAGTGCTGGTGCGAAGGATGCTGA

>Translated drosomycin sequence
MMQIKYLFALFAVLMLVVLGANEADADCLSGRYKGPCAVWDNETCRRVCKEEGRSSG
HCSPSLKCWCEGC

>gi|408407639|sp|B3EWQ0.1|JURTX_AVIJU RecName: Full=U-theraphotoxin-Aju1a; Short=U-TRTX-Aju1a; AltName: Full=Juruin
FTCAISCDIKVNGKPCKGSGEKKCSGGWSCKFNVCVKV

>EMBOSS backtranslation Juruin (Zea mays most likely condon usage)

TTCACCTGCGCCATCAGCTGCGACATCAAGGTGAACGGCAAGCCGTGCAAGGGCAG
CGGCGAGAAGAAGTGCAGCGGCGGCTGGAGCTGCAAGTTCAACGTGTGCGTGAAGG
TG

>Codon optimized juruin
ATGTTCACCTGCGC**g**AT**ttc**CTGCGACATCAAGGTGAACGGCAA**a**CCGTGCAA**a**GGC**tca**
GGCGAGAAGAAGTGC**tca**GGCGGCTGG**tc**CTGCAAGTTCAACGT**t**TGCGTGAAGGTTTG
A

>AF004946.1 Allium cepa antimicrobial protein Ace-AMP1 precursor mRNA, complete cds
AACGAAAATTACGAAATTACATCAATATCTCGAGCC**ATG**GTTCGCGTTGTATCTTTA
CTTGCAGCATCGACCTTCATACTGTTGATTATGATAATCAGCAGTCCGTATGCAAAT
AGTCAGAACATATGCCCAAGGGTTAATCGAATTGTGACACCCTGTGTGGCCTACGGA
CTCGGAAGGGCACCAATCGCCCCATGCTGCAGAGCCCTGAACGATCTACGGTTTGTG
AATACTAGAAACCTACGACGTGCTGCATGCCGCTGCCTCGTAGGGGTAGTGAACCG
GAACCCCGGTCTGAGACGAAACCCTAGATTTCAGAACATTCCTCGTGATTGTCGCAA
CACCTTTGTTCGTCCCTTCTGGTGGCGTCCAAGAATTCAATGCGGCAGGATTAACCTT
ACGGATAAGCTTATATACTTGGACGCTGAGGAA**TGA**AGACTAGGCTCTACTGTTATG
CACTATAGTTTATAGTATATATACTAAATAAAACAGTATGTGCTGTATAATTTGCAA
TATGGACTTATTTATAGCAAGTCCTAATGGTGTCTGCTACTTGGGTCCAGCATTGAG
CACTATATAGGCACTATATAGGGTACTATGGGCTGATTATGATGTCAACGGCGGTAC
TTTATCTTACATAAATAAATAATGGGTTTATCTTGCTTGAAAAAAAAAAAAAAAAAA
AA

>Genscript optimized Ace-AMP
ATGGT**ca**G**g**GT**g**GT**c**TC**gc**T**c**CT**g**GC**t**GC**t**TC**a**ACCTTCAT**c**CT**cc**TGAT**c**ATGAT**c**ATC**tc**CA
G**c**CCGTA**c**GC**t**AA**c**AG**c**CA**a**AACAT**c**TGCCC**tc**GGGT**c**AA**c**AG**a**AT**c**GT**t**AC**g**CC**t**TG**c**GTGG
C**t**TACGG**c**CTCGG**g**AGGGC**g**CCAATCGC**t**CC**t**TGCTGCAGAGC**t**CT**c**AACGA**c**CT**ga**G**a**TT
cGT**c**AA**c**AC**c**AGAAACCT**ca**G**ga**G**a**GCTGC**t**TG**C**a**G**a**T**GCCT**g**GT**t**GG**c**GT**t**GTGAAC**a**G**a**A
ACCC**a**GG**g**CT**cc**G**ga**GAAACCC**gc**G**c**TT**c**CA**a**AACATcCCcaGgGATTG**c**CGCAACACCTT**c**
GT**g**CG**c**CCCTTCTGGTGG**a**G**g**CC**tc**G**c**AT**c**CA**g**TGCGGC**c**GGAT**c**AACCT**g**ACGGA**c**AAGC
T**c**AT**c**TAC**c**TGGA**t**GC**g**GAGGA**g**TGA

>Ace-AMP1 translation
MVRVVSLLAASTFILLIMIISSPYANSQNICPRVNRIVTPCVAYGLGRAPIAPCCRALNDL
RFVNTRNLRRAACRCLVGVVNRNPGLRRNPRFQNIPRDCRNTFVRPFWWRPRIQCGRIN
LTDKLIYLDAEE

>AB012871.1 Wasabia japonica mRNA for gamma-thionin1, complete cds
ATGGCTAAGTTTGCTTCTATCATCGCTCTTCTCTTCGCTGCTCTTGTTCTCTTTTCTGC
TTTTGAAGCACCATCAATGGTGGAAGCGCAGAAGTTGTGCGAGAAGTCAAGTGGGA
CATGGTCAGGAGTCTGTGGAAACAACAATGCGTGCAAGAATCAGTGCATCAACCTT
GAGGGAGCACGACATGGATCTTGCAACTATATCTTCCCATATCACAGATGTATCTGT
TACTTCCCATGT**TAA**TTATTCTACCAAAAAACTTTGGTGCTTAATTAAATATTGTGTG

TGTATTTTACATTTTCTCGTGTGCTTATTCACATTAAATAAGCATATGTCACTCTATG
AGTGACCCCTTATGCATGTACCAAGAATTTCTATGTTGGTTTGTTGTACTAATAAAAT
GTTTTATATGCT

>Genscript optimized Wj-gthionin1
ATGGCTAAGTT**c**GC**c**TC**g**ATCATCGCTCT**c**CT**g**TTCGC**c**GC**g**CT**c**GT**g**CT**g**TT**c**TC**g**GC**g**TT**c**
GA**g**GC**t**CC**c**TCAATGGT**c**GA**g**GCGCA**a**AAG**c**T**c**TGCGAGAAGTC**c**AG**c**GG**c**AC**c**TGGTC**t**G
G**c**GT**t**TG**c**GG**g**AACAACAA**c**GC**c**TGCAAGAA**c**CAGTGCATCAACCT**g**GAGGG**c**GC**ta**G**g**C
A**c**GG**g**TC**c**TGCAACTA**c**ATCTTCCCATA**c**CA**tc**G**c**TG**c**ATCTG**c**TACTTCCC**g**TG**c**TA**a**

>Wj-gthionin1_translation
MAKFASIIALLFAALVLFSAFEAPSMVEAQKLCEKSSGTWSGVCGNNNACKNQCINLEG
ARHGSCNYIFPYHRCICYFPC

>NM_180586.2:162-392 Arabidopsis thaliana hypothetical protein (AT5G36925), mRNA
ATGGCGATGAAGACATCACATGTTCTTCTGCTTTGTTTGATGTTTGTGATTGGTTTTG
TAGAAGCTAGAAGATCAGATACGGGTCCGGATATAAGTACTCCACCATCAGGATCA
TGTGGAGCTTCAATTGCAGAATTCAATTCATCACAAATACTAGCCAAGAGAGCACCA
CCATGTAGACGTCCTCGACTCCAAAACTCAGAAGATGTGACCCACACTACACTTCCT
TGA

>Genscript_ARACIN1
ATGGCGATGAAG**ACcTCcCAc**GTT**CTc**CTG**CTctgcctg**ATG**ttc**GTG**atcggcttcgtcgag**GCT**ag
g**AGAt**ctgacaccgggcca**GAT**atctccacg**CCA**ccttcgggc**TCA**tgcggggccagcatcgcggag**TTC**aactccag
c**CAA**atcctcgct**AAGAGA**gctcctccttgc**AGA**agaccaaggctgcag**AAC**tcggaggacgtcacgcataccacgct
cccg**TGA

>NP_850917.2 hypothetical protein AT5G36925 [Arabidopsis thaliana]
MAMKTSHVLLLCLMFVIGFVEARRSDTGPDISTPPSGSCGASIAEFNSSQILAKRAPPCRR
PRLQNSEDVTHTTLP

Appendix B - Nucleic and Amino Acid Sequences of Interest for Vanillic Acid Work

Lowercase bolded text differs from bioinformatics.org backtranslation of transgenes to *G. max* most likely codon usage.

>gi|27764319|emb|CAD60599.1| unnamed protein product [Podospora anserina]
MPSKLAITSMSLGRCYAGHSFTTKLDMARKYGYQGLELFHEDLADVAYRLSGETPSPC
GPSPAAQLSAARQILRMCQVRNIEIVCLQPFSQYDGLLDREEHERRLEQLEFWIELAHEL
DTDIIQIPANFLPAEEVTEDISLIVSDLQEVADMGLQANPPIRFVYEALCWSTRVDTWERS
WEVVQRVNRPNFGVCLDTFNIAGRVYADPTVASGRTPNAEEAIRKSIARLVERVDVSKV
FYVQVVDAEKLKKPLVPGHRFYDPEQPARMSWSRNCRLFYGEKDRGAYLPVKEIAWA
FFNGLGFEGWVSLELFNRRMSDTGFGVPEELARRGAVSWAKLVRDMKITVDSPTQQQA
TQQPIRMLSLSAAL

> Bioinformatics.org backtranslation of gi|27764319|emb|CAD60599.1| unnamed protein product [Podospora anserina] to a 1101 base sequence of most likely codons (bioinformatics.org_G.m.codonusage).
ATGCCATCTAAGCTTGCTATTACTTCTATGTCTCTTGGAAGATGTTATGCTGGACATT
CTTTACTACTAAGCTTGATATGGCTAGAAAGTATGGATATCAAGGACTTGAACTTTT
TCATGAAGATCTTGCTGATGTTGCTTATAGACTTTCTGGAGAAACTCCATCTCCATGT
GGACCATCTCCAGCTGCTCAACTTTCTGCTGCTAGACAAATTCTTAGAATGTGTCAA
GTTAGAAACATTGAAATTGTTTGTCTTCAACCATTTTCTCAATATGATGGACTTCTTG
ATAGAGAAGAACATGAAAGAAGACTTGAACAACTTGAATTTTGGATTGAACTTGCT
CATGAACTTGATACTGATATTATTCAAATTCCAGCTAACTTTCTTCCAGCTGAAGAA
GTTACTGAAGATATTTCTCTTATTGTTTCTGATCTTCAAGAAGTTGCTGATATGGGAC
TTCAAGCTAACCCACCAATTAGATTTGTTTATGAAGCTCTTTGTTGGTCTACTAGAGT
TGATACTTGGGAAAGATCTTGGGAAGTTGTTCAAAGAGTTAACAGACCAAACTTTGG
AGTTTGTCTTGATACTTTTAACATTGCTGGAAGAGTTTATGCTGATCCAACTGTTGCT
TCTGGAAGAACTCCAAACGCTGAAGAAGCTATTAGAAAGTCTATTGCTAGACTTGTT
GAAAGAGTTGATGTTTCTAAGGTTTTTTATGTTCAAGTTGTTGATGCTGAAAAGCTTA
AGAAGCCACTTGTTCCAGGACATAGATTTTATGATCCAGAACAACCAGCTAGAATGT
CTTGGTCTAGAAACTGTAGACTTTTTTATGGAGAAAAGGATAGAGGAGCTTATCTTC
CAGTTAAGGAAATTGCTTGGGCTTTTTTTAACGGACTTGGATTTGAAGGATGGGTTT
CTCTTGAACTTTTTAACAGAAGAATGTCTGATACTGGATTTGGAGTTCCAGAAGAAC
TTGCTAGAAGAGGAGCTGTTTCTTGGGCTAAGCTTGTTAGAGATATGAAGATTACTG
TTGATTCTCCAACTCAACAACAAGCTACTCAACAACCAATTAGAATGCTTTCTCTTTC
TGCTGCTCTT

>Codon Optimized 3-dehydroshikimate dehydratase
ATGCCATC**c**AAGCT**c**GCTAT**c**ACTTC**c**ATGTCTCTTGGAAGATG**c**TATGCTGGACATTC**c**
TTTAC**c**ACTAAGCT**c**GATATGGCTAG**g**AAGTA**c**GGATA**c**CAAGGACTTGAACT**c**TTTCA

cGAAGATCTTGCTGATGTTGCTTATAG**g**CTTTCTGGAGAAACTCCATCTCCATGTGGA
CCATCTCCAGCTGCTCAACT**c**TCTGCTGCTAGACA**g**AT**c**CTTAG**g**ATGTG**c**CAAGT**g**AG
gAACAT**c**GAAAT**c**GTTTGTCTTCAACCATT**c**TC**c**CAATA**c**GATGGACTTCT**c**GATAGAG
AAGAACATGAA**c**G**t**AGACTTGAACAACTTGA**g**TT**c**TGGAT**c**GAACTTGCTCATGAACT**c**
GATACTGA**c**AT**c**AT**c**CAAATTCCAGC**c**AACTT**c**CTTCCAGCTGAAGAAGT**g**ACTGAAGA
cAT**c**TCTCT**c**AT**c**GT**g**TCTGATCTTCAAGAAGTTGCTGA**c**ATGGGACTTCAAGCTAACC
CACCAATTAG**g**TT**c**GT**g**TA**c**GAAGCTCT**c**TGTTGGTCTACTAGAGTTGA**c**ACTTGGGAA
AG**g**TCTTGGGAAGTTGT**g**CAAAG**g**GT**g**AACAGACC**c**AACTT**c**GGAGTTTGTCT**c**GATAC
cTT**c**AACAT**c**GCTGGAAGAGT**c**TATGCTGATCCAACTGTTGCTTCTGGAAGAACTCCA
AACGCTGAAGAAGCTATTAG**g**AAGTC**c**ATTGCTAGACTTGT**g**GAAAGAGTTGA**c**GT**g**T
CTAAGGT**g**TT**c**TA**c**GTTCAAGTTGTTGATGCTGA**g**AAGCT**c**AAGAAGCCACTTGTTCCA
GGACATAG**g**TT**c**TA**c**GATCCAGAACAACCAGCTAG**g**ATGTCTTGGTCTAG**g**AACTGTA
GACT**c**TT**c**TA**c**GGAGA**g**AAGGATAGAGGAGCTTA**c**CTTCCAGT**g**AAGGAAATTGCTTG
GGC**c**TT**c**TT**c**AACGGACTTGGATT**c**GAAGGATGGGTTTCTCTTGAACT**c**TT**c**AACAGAA
G**g**ATGTCTGATACTGGATTTGGAGTTCCAGAAGAACTTGCTAGAAGAGGAGC**c**GT**g**T
CTTGGGCTAAGCT**c**GTTAGAGA**c**ATGAAGAT**c**ACTGT**g**GATTCTCCAACTCAACAACA
AGCTACTCAACAACCAATTAG**g**ATGCTTTCTCTTTCTGCTGCTCTCTGA

>AAL91506.1 caffeic acid O-methyltransferase II [Nicotiana tabacum]
MESSTKSQIPTQSEEERNCTYAMQLLSSSVLPFVLHSTIQLEVFEILAKSNDTKLSASQIVS
QIPNCTKPEAPTMLNRMLYVLASYSLFTCSIVEDEKNNGGQKRVYGLSQVGKFFVKNEN
GASMGPLLALLQNKVFINSWFELKDAVLEGGVPFDRVHGVHAFEYPKSDPKFNDVFNK
AMINHTTVVMKKILENYKGFENLKTLVDVGGGLGVNLKMITSKYPTIKGTNFDLPHVV
QHAPSYPGVEHVGGDMFESVPEGDAIFMKWILHDWSDSHNLKLLKNCYKALPDNGKVI
VVEAILPVKPDIDTAVVGVSQCDLIMMAQNPGGKERSEEEFRALATEAGFKGVNLICCV
CNFWVMEFCK

>Bioinformatics.org backtranslation of gi|19550748|gb|AF484252.1| Nicotiana tabacum caffeic acid O-methyltransferase II gene, complete cds (bioinformatics.org Glycine max codon usage)
ATGGAATCTTCTACTAAGTCTCAAATTCCAACTCAATCTGAAGAAGAAAGAAACTGT
ACTTATGCTATGCAACTTCTTTCTTCTTCTGTTCTTCCATTTGTTCTTCATTCTACTATT
CAACTTGAAGTTTTTGAAATTCTTGCTAAGTCTAACGATACTAAGCTTTCTGCTTCTC
AAATTGTTTCTCAAATTCCAAACTGTACTAAGCCAGAAGCTCCAACTATGCTTAACA
GAATGCTTTATGTTCTTGCTTCTTATTCTCTTTTTACTTGTTCTATTGTTGAAGATGAA
AAGAACAACGGAGGACAAAAGAGAGTTTATGGACTTTCTCAAGTTGGAAAGTTTTT
TGTTAAGAACGAAAACGGAGCTTCTATGGGACCACTTCTTGCTCTTCTTCAAAACAA
GGTTTTTATTAACTCTTGGTTTGAACTTAAGGATGCTGTTCTTGAAGGAGGAGTTCCA
TTTGATAGAGTTCATGGAGTTCATGCTTTTGAATATCCAAAGTCTGATCCAAAGTTTA
ACGATGTTTTTAACAAGGCTATGATTAACCATACTACTGTTGTTATGAAGAAGATTC
TTGAAAACTATAAGGGATTTGAAAACCTTAAGACTCTTGTTGATGTTGGAGGAGGAC
TTGGAGTTAACCTTAAGATGATTACTTCTAAGTATCCAACTATTAAGGGAACTAACT
TTGATCTTCCACATGTTGTTCAACATGCTCCATCTTATCCAGGAGTTGAACATGTTGG
AGGAGATATGTTTGAATCTGTTCCAGAAGGAGATGCTATTTTTATGAAGTGGATTCT
TCATGATTGGTCTGATTCTCATAACCTTAAGCTTCTTAAGAACTGTTATAAGGCTCTT
CCAGATAACGGAAAGGTTATTGTTGTTGAAGCTATTCTTCCAGTTAAGCCAGATATT
GATACTGCTGTTGTTGGAGTTTCTCAATGTGATCTTATATGATGGCTCAAAACCCAG

GAGGAAAGGAAAGATCTGAAGAAGAATTTAGAGCTCTTGCTACTGAAGCTGGATTT
AAGGGAGTTAACCTTATTTGTTGTGTTTGTAACTTTTGGGTTATGGAATTTTGTAAG

>Codon optimized O-Methyltransferase
ATGGAATC**c**TCTAC**c**AAGTCTCA**g**ATTCCAACTCAATCTGAAGAAGA**g**AGAAACTGTA
C**c**TATGCTATGCAACTTCT**c**TCTTCTTCTGTTCTTCCATT**c**GTTCT**c**CATTC**c**ACTAT**c**CA
ACTTGAAGT**g**TT**c**GAAAT**c**CT**c**GCTAAGTCTAACGATAC**c**AAGCT**c**TCTGCTTCTCA**g**AT
cGTTTC**c**CAAATTCCAAACTGTAC**c**AAGCCAGAAGCTCCAAC**c**ATGCT**c**AACAGAATG
CTTTA**c**GTTCTTGCTTC**c**TATTC**c**CT**c**TT**c**ACTTGTTC**c**ATTGT**g**GAAGATGA**g**AAGAACA
ACGGAGGACA**g**AAGAG**g**GTTTATGGACTTTCTCAAGTTGG**c**AAGTT**c**TT**c**GT**g**AAGAA
CGA**g**AACGGAGCT**agc**ATGGGACCACTTCTTGCTCTTCT**c**CA**g**AACAAGGT**g**TT**c**AT**c**AA
CTCTTGGTTTGA**g**CTTAAGGATGCTGTTCTTGAAGGAGGAGTTCCATT**c**GATAGAGTT
CATGGAGTTCATGCTTT**c**GAATATCCAAAGTCTGATCC**c**AAGTT**c**AACGA**c**GT**g**TT**c**AA
CAAGGC**c**ATGAT**c**AACCATAC**c**ACTGTTGT**g**ATGAAGAAGAT**c**CTTGA**g**AACTA**c**AAGG
GATT**c**GA**g**AACCT**c**AAGACTCTTGTTGATGTTGGAGGAGGACTTGGAGT**g**AACCT**c**AA
GATGAT**c**AC**c**TC**c**AAGTA**c**CCAAC**c**ATTAAGGGAAC**c**AACTT**c**GATCTTCCACATGTTG
TTCAACATGCTCCATC**c**TATCCAGGAGTTGAACATGTTGGAGG**c**GA**c**ATGTT**c**GAATC
TGTTCCAGAAGGAGATGC**c**AT**c**TT**c**ATGAAGTGGATTCT**c**CATGA**c**TGGTCTGATTC**c**C
ATAACCTTAAGCT**c**CT**c**AAGAACTG**c**TA**c**AAGGCTCTTCCAGA**c**AACGGAAAGGTTAT**c**
GTTGTTGAAGCTAT**c**CTTCCAGT**g**AAGCCAGA**c**AT**c**GATACTGCTGTTGTTGGAGTTTC
TCAATGTGATCT**c**AT**c**ATGATGGCTCA**g**AACCCAGGAGGAAAGGAA**c**G**c**TCTGAAGAA
GA**g**TTT**c**G**t**GCTCTTGCTACTGAAGCTGG**c**TT**c**AAGGGAGT**g**AACCT**c**AT**c**TGTTG**c**GT**c**T
G**c**AACTT**c**TGGGTTATGGA**g**TT**c**TG**c**AAGTGA

>gb|KJ787649.1|:1-216 Synthetic construct 5-enol-pyruvylshikimate-3-phospate synthase (EPSPS) chloroplast transit peptide derived from Petunia x hybrida,
ATGGCCCAGATTAACAATATGGCACAAGGCATCCAGACTCTCAATCCCAACTCTAAT
TTCCATAAGCCACAGGTTCCTAAAAGCTCTAGCTTTCTTGTCTTCGGTTCCAAGAAGT
TGAAGAACTCTGCTAATTCTATGTTGGTGCTTAAGAAAGATTCAATCTTCATGCAAA
AGTTCTGTTCATTCAGAATTTCTGCTTCCGTGGCCACTGCATGC

>Codon optimized Petunia x hybrida EPSPS chloroplast transit peptide
ATGGCCCAGAT**c**AACAA**c**ATGGCACAAGGCATCCAGACTCTCAATCCCAACTC**c**AA**c**T
TCCATAAGCCACAGGTTCCTAA**g**AGCTCTAGCTTTCT**c**GTCTTCGGTTCCAAGAAGTT
GAAGAACTCTGCTAA**c**TC**c**ATG**c**T**t**GTGCT**c**AAGAA**g**GATTC**c**ATCTTCATGCA**g**AAGTT
CTGTTC**c**TTCAG**g**AT**c**TCTGCTTCCGTGGCCACTGCATGC

Appendix C - Primers used in this work

Primer Name	Target	Sequence (5'→3')
Defensin GOI primers		
Zea-F1	Zeamatin_JB CDS	CAATCGTGGGCATCTTCGTC
Zea-R1	Zeamatin_JB CDS	ACCTTGTAGTTGGTTCCGGC
Zea-F2	Zeamatin_JB CDS	CTTCACGGTGGTGAACCAGT
Zea-R2	Zeamatin_JB CDS	CTGCCCCTTGAAGTACCTGG
ZeaM-F	Zeamatin_Genscript CDS	CTGTTTTCACGGTGGTCAAC
ZeaM-R	Zeamatin_Genscript CDS	CATCGTCCTTAGGGTAAGAGTAAG
Dros-F1	Drosomycin CDS	CCCTCTTCGCTGTCCTGATG
Dros-R1	Drosomycin CDS	TCCCTCCTCCTTGCAAACAC
Dros-F2	Drosomycin CDS	AGTACTTGTTCGCCCTCTTCG
Dros-R2	Drosomycin CDS	CCTTGCAAACACGACGACAG
Jur-F1	Juruin CDS	CTGCGACATCAAGGTGAACG
Jur-R1	Juruin CDS	TTGAACTTGCAGGACCAGCC
Jur-F2	Juruin CDS	CCTGCGACATCAAGGTGAAC
Jur-R2	Juruin CDS	TGAACTTGCAGGACCAGCC
Ace-F	Ace AMP CDS	GTACGCTAACAGCCAAAACATCT
Ace-R	Ace AMP CDS	ATCCAGGTAGATGAGCTTGTCC
Ara-F	Aracin CDS	GATGAAGACCTCCCACGTTCT
Ara-R	Aracin CDS	GTGACGTCCTCCGAGTTCTG
Thio-F	Glutathionin CDS	GCTAAGTTCGCCTCGATCAT
Thio-R	Glutathionin CDS	GATGGTATGGGAAGATGTAGTTG
Vanillic acid GOI primers		
Night-F1	3DSD CDS	TGCAAAGGGTGAACAGACCC
Night-R1	3DSD CDS	AGCTGGTTGTTCTGGATCGT
Night-F2	3DSD CDS	GTGCCAAGTGAGGAACATCG
Night-R2	3DSD CDS	GCTTGAAGTCCCATGTCAGC
Hawk-F1	COMT CDS	GAGAACGGAGCTAGCATGGG
Hawk-R1	COMT CDS	ATTCGAACATGTCGCCTCCA
Hawk-F2	COMT CDS	CACCTCCAAGTACCCAACCA
Hawk-R2	COMT CDS	GCTTCAGTAGCAAGAGCACG
gDNA confirmation/mRNA check primers		
Rib-F	*G.max* 40S ribosome	CTAAGATGCAGAACGAGGAAGG
Rib-R	*G.max* 40S ribosome	GAGAGCAAAAGTGGAGAAATGG
Tub-F	*A. triticum* Tubulin	ATCTGTGCCTTGACCGTATCAGG

Tub-R	*A. triticum* Tubulin	GACATCAACATTCAGAGCACCATC
AtTub9-F	*A. thaliana* Tubulin	GGCAAATACGTTCCTCGTGC
AtTub9-R	*A. thaliana* Tubulin	CAGGGAACCGAAGACAGCAT

Subcloning and sequencing primers

GmUBI se-F	*G.max* Ubiquitin promoter	TCAGATCCGTTGACAAAAAGC
CaMV35SF	CaMV promoter	GCACAATCCCACTATCCTTCGCAA
Tnos-R	NOS terminator	CCAGTGAATTCCCGATCTAGT
NOS-R	NOS terminator	CAGGATTCAATCTTAAGAAACTT
oriF	Origin of replication	GGAGAAAGGCGGACAGGTAT
oriR	Origin of replication	GCTTCCTCGCTCACTGACTC
6010MCS-F	pME6010 MCS	CAAAGCCACGTTGTGTCTCA
6010MCS-R	pME6010 MCS	GGAGAAAACTCACCGAGGCA
M13-F	General sequencing	GTAAAACGACGGCCAG
M13-R	General sequencing	TCACACAGGAAACAGCTATGAC
KpnI_TEV_Dros44-F	Const. MBP-Drosomycin	TAAGCGGTACCGAAAATCTGTATTTT CAGGGCGACTGCCTGTCCGGAAGGT ATAAGGGT
Dros_SacI-R	Const. MBP-Drosomycin	TAAGCGAGCTCTTATCAGCATCCTTC GCACCAGCAC
KpnI_TEV_Jur-F	Const. MBP-Juruin	TAAGCGGTACCGAAAATCTGTATTTTC AGGGCTTCACCTGCGCGATTTCCTGCG
Jur_SacI-R	Const. MBP-Juruin	TAAGCGAGCTCTTATCAAACCTTCAC GCAAACGTTG
Night_Hind3-F	Const. pME6010NH	TAAGCAAGCTTAATGCCATCCAAGCTC GCTATC
Night_Hind3-R	Const. pME6010NH	TAAGCAAGCTTTCAGAGAGCAGCAGA AAGAG
Lac_Sph1-F	Const. pME6010NH	TAAGCGCATGCTTTCCCGACTGGAAA GCGGGC
SacI-RBS-Hawk-F	Const. pME6010NH	TAAGCGAGCTCAAAGAGGAGGAATAC TAGATGGAATCCTCTACCAAGTCTCAG
Hawk_SacI-R	Const. pME6010NH	TAAGCGAGCTCTCACTTGCAGAACTC CATAACC
KpnI-Gmubi-F	Const. pBI121NH	TAAGCGGTACCGCATGCGGGCCCAAT ATAACA
KpnI-TNOS-R	Const. pBI121NH	TAAGCGGTACCGAATTCTCATGTTTGA CAGCT

Selectable marker primers

Hyg-F1	Hygromycin resistance gene	AAAAGTTCGACAGCGTCTCC
Hyg-R1	Hygromycin resistance gene	GATGTTGGCGACCTCGTATT
Bar-5'	Bar gene	GAAGTCCAGCTGCCAGAAAC
Bar-3'	Bar gene	AGTCGACCGTGTACGTCTCC

RT-qPCR specific primers		
qPCR-zeaM-F		GTCAGCCGCGGAACGATTG
qPCR-thio-F		AACAACGCCTGCAAGAACC
qPCR-arc-R		GCCCGGTGTCAGATCTCCTA
qPCR-ace-F		AAACATCCCCAGGGATTGCC
qTaEFα-F	Wheat elongation factor alpha	CAGATTGGCAACGGCTACG
qTaEFα-R	Wheat elongation factor alpha	CGGACAGCAAAACGACCAAG
qTaGAPDH-F	Wheat GAPDH	TTCAACATCATTCCAAGCAGCA
qTaGAPDH-R	Wheat GAPDH	CGTAACCCAAAATGCCCTTG
qTaCyclop-F	Wheat cyclophilin	CAAGCCGCTGCACTACAAGG
qTaCyclop-R	Wheat cyclophilin	AGGGGACGGTGCAGATGAA
qTaActin-F	Wheat actin	GACAATGGAACCGGAATGGTC
qTaActin-R	Wheat actin	GTGTGATGCCAGATTTTCTCCAT

Appendix D - Plasmids used and generated for this work

Plasmid	Backbone	Synonymous name	Description
pUC57	-		Bacterial cloning/expression vector
pAHC17	-		Monocot expression vector with *Zea mays* ubiquitin promoter
pAHC20	-		Monocot expression vector conferring glufosinate tolerance
pGmubi	-		Dicot expression vector with *Glycine max* ubiquitin promoter
pHyg	-		Dicot expression vector conferring hygromycin tolerance
pLic-MBP-APETx2	-		Bacterial expression vector for MBP fusion expression driven by lacI promoter
pDZ2087	-		Bacterial expression vector for TEV protease production
pME6010	-		Broad host range bacterial expression vector
pBICaMV	-		Agrobacterium binary vector; transgene expression via CaMV35s promoter
pJBdef1	pUC57		Cloning vector containing synthetic Juruin, Drosomycin, and Zeamatin CDSs, generated by Genscript
pGm3DSD	pUC57	pGmNight	Cloning vector containing synthetic *Podospora anserine* 3DSD CDS, generated by Genscript
pGmCOMT	pUC57	pGmHawk	Cloning vector containing synthetic *Nicotiana tobacum* COMT CDS, generated by Genscript
Constructed plasmids			
pGmubi3DSD	pGmubi	pGmubiNight	*P. anserine* 3DSD CDS in pGmubi
pGmubiCOMT	pGmubi	pGmubiHawk	*N. tobacum* COMT CDS in pGmubi
pGmubiZea	pGmubi	pZeaGubi	Zeamatin CDS in pGmubi
pGmubiDros	pGmubi	pDrosGubi	Drosomycin CDS in pGmubi
pGmubiJur	pGmubi	pJurGubi	Juruin CDS in pGmubi
pDros17	pAHC17		Drosomycin CDS in pAHC17
pJur17	pAHC17		Juruin CDS in pAHC17
p121Ara	pBICaMV		Aracin CDS in pBICaMV
p121Jur	pBICaMV		Juruin CDS in pBICaMV
p121Thio	pBICaMV		Gamma-Thionin CDS in pBICaMV
p121Zea	pBICaMV		Zeamatin CDS in pBICaMV
p121Pa3DSD	pBICaMV	p121N	*P. anserine* 3DSD CDS in pBICaMV
p121NtCOMT	pBICaMV	p121H	*N. tobacum* COMT CDS in pBICaMV
p121Pa3dsdCOMT	pBICaMV	p121nH	Intermediate vector for expression of VA CDSs; COMT expression driven by *G. max* ubiquitin promoter
pLicDros	pLic-MBP-APETx2		*Drosomycin* in pLic-MBP-APETx2
pLicJur	pLic-MBP-APETx2		*Juruin* in pLic-MBP-APETx2

pMalJur	pLic-MBP-APETx2		MBP sequence removed with MalE signal sequence fused to *Juruin*
pLic-MBP	pLic-MBP-APETx2		Experimental control vector for production of MBP fusion only
pDZ2089	pDZ2087	pDZ2087ΔTEV	Negative control vector with TEV CDS removed
pUC57m	pUC57	pUC57mod, p57Δ	ΔlacZ60-63GAT of pUC57
p57Pa3DSD	pUC57	pUC57Night, pUC57N	LacZ-3DSD fusion expression for *E. coli*; intermidate vector for p6010VA construction
p6010Pa3DSD	pME6010	p6010N, pME6010N	LacZ-3DSD fusion broad host range expression vector; intermidate vector for p6010VA construction
p6010VA	pME6010	p6010NrH	3DSD-COMT operon broad host range expression vector; expression via lac promoter
pME6010-GFP	pME6010		GFP broad-host range expression vector; expression driven via Tc promoter

Appendix E - Abbreviations

3-DHS	3-dehydroshikimic acid
3-DSD	3-dehydroshikimate dehydratase
AA	Amino acid
AcTEV	Tobacco Etch Virus protease (ThermoFisher)
ANOVA	Analysis of variance
Ara	Aracin1
bla	β-lactamase
bp	Base pair
BW	Bobwhite (wheat variety)
CB	Common bunt
cDNA	Complementary DNA
CDS	Protein coding sequence
COMT	Catechol o-methyltransferase
CRD	Completely random design
DNA	Deoxyribonucleic acid
Dros	Drosomycin
DON	Deoxynivalenol
FHB	Fusarium head blight
EDTA	Ethylenediamine tetraacetic acid
EFα	Elongation factor alpha
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
gDNA	Genomic DNA
GM	Genetically modified
GOI	Gene of interest
GSH	Glutathione and
GSSG	Glutathione disulfide
GUS	β-glucuronidase
IC_{50}	50% growth inhibitory concentration
IPTG	Isopropyl β-D-1-thiogalactopyranoside
J2	Second-stage juvenile (SCN)
Jur	Juruin
LB	Luria-Bertani (media)
LMPE	Low molecular weight protein extract
LTP	Lipid transfer protein
MCS	Multiple cloning site
mRNA	Messenger RNA
MW	Molecular weight
mya	Million years ago
NaCl	Sodium chloride
NtOMT	*Nicotiana tabacum* O-methyltransferase
MALDI-TOF	Matrix assisted laser desorption/ionization-time of flight
MBP	Maltose binding protein
OD_{600}	Optical density at 600 nm

OMT	O-methyltransferase
ORF	Open reading frame
PDA	Potato dextrose agar
PDB	Potato dextrose broth
PCR	Polymerase chain reaction
PCA	Protocatechuic acid
Ptr	*Pyrenophora tritici-repentis*
PMSF	Phenylmethylsulfonyl fluoride
QTL	Quantitative trait loci
rDros	Recombinant drosomycin
rJur	Recombinant juruin
RCBD	Randomized complete block design
RH	Relative humidity
RNA	Ribonucleic acid
RNAi	RNA interference
RP-HPLC	Reverse phase-high performance liquid chromatograph
RT	Room temperature
RT-PCR	Reverse transcription polymerase chain reaction
RT-qPCR	Real-time quantitative PCR
SAM	S-adenosyl-L-methionine
SCN	Soybean cyst nematode
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SQ	Starting quantity
T-DNA	Transfer DNA
TC	Tissue culture (control treatment)
TE	Transposable element
TEVp	Tobacco Etch Virus protease
Thio	Gamma-thionin
TIR	Tandem inverted repeat
T_m	Melting temperature
Tnos	Nopaline synthase terminator
TS	Tan spot
VA	Vanillic acid
W/T	Wild type
Zea	Zeamatin