DISSERTATION

FUNCTIONAL GENOMICS APPROACHES TO CEREAL–APHID INTERACTIONS

Submitted by

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ABSTRACT

FUNCTIONAL GENOMICS APPROACHES TO CEREAL–APHID INTERACTIONS

Plants and aphids are locked in a ceaseless molecular arms race. This has resulted in the evolution of molecular surveillance mechanisms in plants to detect the presence of phloem feeding insects and activate a large and dynamically regulated repertoire of defense responses. In turn, aphids have evolved sophisticated strategies to evade detection, suppress defense responses and alter plant metabolism. In this study, several different functional genomics approaches were employed to gain insight into the molecular components contributing to both aphid virulence and the resistance responses of cereals. To date, a myriad of plant genes have been identified as differentially regulated during responses to aphid attack. However, determining their exact function in plant defense has remained a particularly perplexing endeavor. In particular, progress in understanding the mechanism of wheat resistance to aphids has been hampered by a lack of mutant germplasm collections and the fact that wheat is not readily amenable to transformation.

Virus-induced gene silencing (VIGS) technology has emerged as a viable reverse genetics approach in cereal crops. This study is the first report on the successful use of VIGS to investigate genes involved in cereal–insect interactions. The *WRKY53* gene was selected as a candidate likely to be important in the wheat defense response against the Russian wheat aphid. This report details the use of recombinant barley stripe

mosaic virus (BSMV) to target and silence a *WRKY53* transcription factor and an inducible phenylalanine ammonia-lyase (*PAL*) gene, both predicted to contribute to aphid defense in a genetically resistant wheat line. After inoculating resistant wheat with the VIGS constructs, transcript abundance was reduced to levels similar to that observed in susceptible wheat genotypes. Notably, the level of *PAL* expression was also suppressed by the *WRKY53* construct, suggesting that these genes operate in the same defense response network. Interestingly, both silenced lines exhibited decreased levels of H_2O_2, a component of the oxidative burst, in response to aphid feeding. Both knockdowns exhibited a susceptible phenotype upon aphid infestation, and aphids feeding on silenced plants exhibited a significant increase in fitness compared to aphids feeding on control plants. Altered plant phenotype and changes in aphid behavior after silencing imply that *WRKY53* and *PAL* play key roles in generating a successful resistance response.

Very little is known about the structure, regulation and function of the wheat *WRKY53* gene. Therefore, the genomic region encoding the wheat *WRKY53* gene and its *cis*-acting regulatory elements was characterized, and other genetic components of the *WRKY53* transcriptional network were determined, including regulators upstream from *WRKY53* in the defense signal cascade as well as downstream target genes.

A PCR-based approach was used to obtain the 1.2 kb promoter region as well as a full-length genomic clone of the wheat *WRKY53* gene, which is composed of five exons, similar to the rice ortholog. The promoter region of wheat *WRKY53* has three W-box WRKY-binding domains, consistent with the distribution of W-boxes in orthologs from other species. Sequence analysis of *WRKY53* alleles from different Asian landraces with different levels of aphid resistance found that the gene is largely functionally constrained, with very few amino acid substitutions between accessions. Based on the few substitutions that were observed, especially in the transactivating N-terminal region

of the protein, the accessions could be grouped according to aphid resistance, as opposed to geographic center of origin. Based on gene coexpression networks in rice and the presence of W-boxes in their 1 kb promoter regions, a chitinase, a peroxidase and a receptor kinase were predicted to be likely downstream targets of the WRKY53 transcription factor, and the ability of recombinant WRKY53 protein to interact with these promoter regions was tested using an electrophoretic mobility shift assay. Yeast two-hybrid analyses using a rice cDNA library enriched for pathogen-responsive genes were performed to discover novel protein–protein interactions involving WRKY53. A microsomal glutathione *S*-transferase showed a strong interaction with the WRKY53 protein, offering further support for the regulatory role of *WRKY53* during the oxidative burst. A yeast one-hybrid approach was used to discover novel proteins able to interact with the promoter region of *WRKY53* and modulate its function. A calmodulin-related calcium sensor protein, an ultraviolet-B repressible protein and a DUF584 protein were discovered as possible upstream regulators of the *WRKY53* network. Collectively, the data suggests that *WRKY53* acts as a transcriptional regulator of several defense-related pathways in cereals.

Since aphids use their saliva to modulate plant defense responses, the salivary proteins secreted by two Russian wheat aphid biotypes were compared. Saliva was collected from biotypes RWA1 and RWA2 using feeding cages and an artificial sucrose diet and compared using fluorescence differential gel electrophoresis (DIGE). Differentially expressed proteins were excised from 2D gels and subjected to mass spectrometry. Proteins were assigned identities via homology to gene models in the *Acyrthosiphon pisum* genome sequence and a database of *Schizaphis graminum* short sequence reads. The analysis uncovered several proteins previously reported from aphid saliva, including glucose dehydrogenase, aminopeptidase and apolipophorin. However, proteins of bacterial origin, inoculated into the artificial diet by the aphids, formed the

largest contribution to the complex protein patterns observed. Subsequent iTRAQ (isobaric tags for relative and absolute quantification) and MuDPIT (multidimensional protein identification technology) analysis suggested that the contribution of aphid-derived proteins to the total protein pool from the artificial diets was small, but that the number of aphid-specific proteins identified corresponds to previous reports for salivary proteins collected from artificial diets. It is hypothesized that all aphid species share a few common proteins in their secreted saliva and are able to modulate the exact composition to some extent. Based on the discrepancy between secreted proteins and proteins with secretion signals isolated from salivary glands, it is speculated that some proteins may be specific to E2 salivation, which was not collected using the described experimental setup. Russian wheat aphids likely inoculate plants with microbes or microbial peptides, thereby eliciting inappropriate stress responses that accelerate disease symptoms such as chlorosis and help to mobilize nitrogen into the phloem.

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"What is called the scientific method is only imagination set within bounds."
—Liberty Hyde Bailey, 1903

"Science is the art of our time. Science has several rewards, but the greatest is that it is the most interesting, difficult, pitiless, exhilarating, and beautiful pursuit that we have yet found."
—Horace Freeland Judson, 2004

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CHAPTER 1:

INTRODUCTION

Aphid resistance in plants: at the interface of pathogen and insect resistance

The interactions between plants and insects are complex and dynamic. Being sessile, plants are unable to escape from predators and have evolved diverse physical barriers and highly sophisticated biochemistry to defend themselves. Plants perceive the presence of an insect threat, either through metabolic changes in injured tissues or through the binding of the molecular signatures of attack to specifically evolved plant receptors, and initiate an appropriate response (Mithöfer and Boland, 2008). In turn, insects have evolved specialized mechanisms to evade perception, downregulate plant defense responses and gain maximum nutrition from their host.

Aphids are phloem-feeding insects and, in contrast to chewing insects such as caterpillars, have a much more intimate association with their plant host that lends itself well to comparisons with plant–pathogen interactions. In fact, we observe significant overlap in the aphid and pathogen defense repertoires. This includes the general perception of biotic attack to induce basal resistance (Boller and He, 2009), and the use of gene-for-gene interactions (Flor, 1971) between plant *R* genes and insect/pathogen *avr* genes in a race- or species-specific manner to elicit an effector-triggered immunity. These responses range from restructuring of the cellular architecture and the production of xenotoxic phytochemicals, to the oxidative burst and hypersensitive cell death around the site of feeding or infection (Dangl and Jones, 2001; Flor, 1971; Goggin, 2007; Kaloshian, 2004; Kaloshian and Walling, 2005). This level of complexity requires highly

coordinated gene expression and several plant signaling pathways are involved. Aphids feeding on plants not only induce the salicylic acid (SA) pathway activated in response to biotrophic pathogens, but also some components of the jasmonic acid/ethylene (JA/ET) pathways associated with necrotrophic pathogens and chewing insects (Botha *et al.*, 2005; Kempema *et al.*, 2007). This activation of pathways generally regarded as antagonistic (Chisholm *et al.*, 2006) is a hallmark of aphid resistance (Botha *et al.*, 2010; Kaloshian, 2004; Smith and Boyko, 2007).

Transcription factors are important regulators of plant disease resistance

The last decade of research in plant–aphid interactions has made huge advances toward discovering the plant genes that are involved in aphid resistance. Aphids probe intercellularly before reaching the sieve elements of the phloem (Botha *et al.*, 1975), and in doing so induce the apoplastic accumulation of pathogenesis-related proteins such as chitinase and peroxidase (Van der Westhuizen *et al.*, 1998). Therefore, initial work focused mainly on enzyme activities in the apoplast. However, the recent ubiquity of high throughput transcriptomics tools has greatly accelerated the rate at which plant genes differentially expressed in response to aphid feeding have been discovered. The results from these studies can be compiled into a very extensive list of differentially regulated genes spanning many diverse functions and physiological processes, including photosynthetic regulation, signaling cascades, transcription factors, cell wall modifying enzymes, and genes involved in the oxidative burst and hypersensitive cell death response (Botha *et al.*, 2010; Boyko *et al.*, 2006; De Vos and Jander, 2009; Gutsche *et al.*, 2009; Li *et al.*, 2008; Liu *et al.*, 2011; Zaayman *et al.*, 2009). However, differential regulation of a gene does not necessarily imply the requirement of that specific gene for effective resistance. The particular roles of most genes seemingly involved in aphid

resistance are unclear. This study addressed some of these uncertainties by taking a functional genomics approach to cereal–aphid interactions.

In Chapter 2, *WRKY53*, a transcription factor of wheat (*Triticum aestivum* L.), was selected as a candidate gene that likely has an important regulatory function in defense against the Russian wheat aphid (*Diuraphis noxia* Kurdjumov). A transient loss-of-function experiment was designed to address several pertinent questions regarding the role of *WRKY53* in enhancing or modulating the *D. noxia* resistance response of wheat. In particular, this assay addressed the question of whether this gene is required for effective resistance, or merely coregulated during the transcriptional reprogramming associated with defense. The phenotypic traits that are under the control of the gene were ascertained, and it was also determined whether aphids would find *WRKY53* silenced plants more suitable hosts for feeding and reproduction. Subsequently, the transcription factor and its function were characterized in more detail.

In Chapter 3, the gene features and *cis*-acting regulatory elements of *WRKY53* were determined. This allowed additional questions about the function of *WRKY53* to be addressed, including which *trans*-acting genes interact with its promoter and how their roles in plant defense might be linked. Downstream genes targeted for regulation by this transcription factor were identified and it was determined whether any other proteins interact with WRKY53, thereby affecting its activity.

Aphid saliva is the source of virulence factors and plant defense elicitors

When aphids establish a feeding site on a plant, they insert their thin, flexible styles into the leaf tissue, probing intercellularly until the sieve elements of the phloem are reached. Plants have evolved mechanisms to detect this attack, including the deposition of coagulating proteins onto and callose collars around the pores connecting sieve elements in an attempt to plug them and stop the flow of nutrients. In turn, aphids have evolved

mechanisms to suppress this plugging response (Will and Van Bel, 2006). Aphids are also able to prevent Ca^{2+} influx and thus inhibit the induction of signaling cascades. The movement of Ca^{2+} into the cell can be prevented by a seal formed from the aphid's gel-like saliva over the penetration wound (Will and Van Bel, 2006), or by chelation of Ca^{2+} by salivary proteins (Will *et al.*, 2007). Feeding damage caused by *D. noxia* produces idiosyncratic symptoms, including longitudinal chlorotic streaking and osmotic dysregulation leading to leaf rolling (Burd and Burton, 1992). The saliva of *D. noxia* in particular is able to induce accelerated leaf senescence and chloroplast breakdown during compatible interactions (Botha *et al.*, 2006; Franzen *et al.*, 2007). Unlike many other agronomically important species, *D. noxia* are not vectors for viral transmission (Halbert *et al.*, 1992). This trait, coupled with their narrow host range and striking disease phenotype, distinguishes *D. noxia* from other aphids including the pea aphid, *Acyrthosiphon pisum*, which is a better-studied model species with an available genome sequence (The International Aphid Genomics Consortium, 2010). There is a paucity of scientific reporting on the composition of *D. noxia* saliva (Cooper *et al.*, 2010, 2011), and although attempts have been made (Lapitan *et al.*, 2007), specific protein elicitors of cereal resistance responses have not been identified to date. The identification of differentially expressed proteins from the sialome (salivary proteome) of different biotypes of *D. noxia* is therefore of primary interest to researchers in the field.

In Chapter 4, salivary proteins from aphid biotypes RWA1 and RWA2 were collected and identified using a 2D difference gel electrophoresis DIGE coupled to peptide identification using mass spectrometry (Cilia *et al.*, 2009). This allowed questions to be addressed such as whether the salivary proteins of biotypes differ, and whether those differences are qualitative or quantitative. Since identified proteins may have an important function as virulence determinants or elicitors of defense responses, it

would be of interest to ascertain their identity and possible function during aphid feeding and plant defense response induction.

A diverse array of functional genomics approaches were used to demonstrate that WRKY-based transcriptional networks are involved in aphid resistance responses, and some of the upstream and downstream components of this signaling network were determined. In particular, this study focused on *WRKY53* to show that it is required for defense gene-mediated aphid resistance in wheat. Some of the *trans*-acting genes that interact with the *WRKY53* promoter region and the downstream genes this transcription factor targets for gene regulation were identified. The insect component of cereal–aphid interactions was also approached, using a proteomics analyses to identify the proteins present in the secreted saliva of the Russian wheat aphid, and also to compare the composition of secreted saliva between aphid biotypes of different virulence.

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CHAPTER 2:

VIGS OF *WRKY53* AND AN INDUCIBLE *PAL* GENE IN WHEAT

SUMMARY

Although several wheat genes differentially expressed during the Russian wheat aphid resistance response have recently been identified, their requirement for and specific role in resistance remain unclear. Progress in wheat–aphid interaction research is hampered by inadequate collections of mutant germplasm and difficulty in transforming hexaploid wheat. Virus-induced gene silencing (VIGS) technology is emerging as a viable reverse genetics approach in cereal crops. However, the potential of VIGS for determining aphid defense gene function in wheat has not been evaluated. We report on the use of recombinant barley stripe mosaic virus (BSMV) to target and silence a *WRKY53* transcription factor and an inducible phenylalanine ammonia-lyase (*PAL*) gene, both predicted to contribute to aphid defense in a genetically resistant wheat line. After inoculating resistant wheat with the VIGS constructs, transcript abundance was reduced to levels similar to that observed in susceptible wheat. Notably, the level of *PAL* expression was also suppressed by the *WRKY53* construct, suggesting that these genes operate in the same defense response network. Both knockdowns exhibited a susceptible phenotype upon aphid infestation, and aphids feeding on silenced plants exhibited a significant increase in fitness compared to aphids feeding on control plants. Altered

plant phenotype and changes in aphid behavior after silencing imply that *WRKY53* and *PAL* play key roles in generating a successful resistance response. This study is the first report on the successful use of VIGS to investigate genes involved in wheat–insect interactions.

INTRODUCTION

Confirmation of defense gene function: beyond gene discovery

Recent advances in wheat (*Triticum aestivum* L.) functional genomics have identified hundreds of transcripts differentially expressed during the resistance response against the Russian wheat aphid (*Diuraphis noxia* Kurdjumov). Expression profiling studies such as cDNA-AFLPs (Zaayman *et al.*, 2009), SSH cDNA libraries (Boyko *et al.*, 2006) and microarray analysis (Botha *et al.*, 2006; Botha *et al.*, 2010; Gutsche *et al.*, 2009; Lapitan *et al.*, 2008; Smith *et al.*, 2010) constitute a transcriptomics resource that has remained largely underutilized. These techniques rely heavily on sequence homology analysis and functional inference from better characterized plant-pathogen pathways. They are also unable to discriminate between coregulated transcripts and those directly responsible for the resistance phenotype. It is therefore essential that information garnered from these studies be verified with complementary techniques before a model of wheat resistance to aphids can be developed. Unfortunately, verifying that these candidate genes are not only involved in but required for the development of aphid resistance has been impeded by the idiosyncrasies of studying wheat. Cultivated wheat is hexaploid with a large genome; this makes mutational analysis difficult, and currently no mutant diploid germplasm collections are available (Cakir and Scofield, 2008). T-DNA transformation has also proved challenging (Scofield *et al.*, 2005).

The virus-induced gene silencing approach

Virus-induced gene silencing (VIGS) is emerging as a suitable functional analysis approach in cereals. It is based on a form of post-transcriptional gene silencing (PTGS) where the presence of double-stranded viral RNA activates a host defense mechanism: dsRNA is cleaved by a Dicer-like enzyme into small interfering RNAs (siRNAs), which in turn direct the RNA-induced silencing complex (RISC) to degrade any RNA with sufficient homology (Bonnet *et al.*, 2006; Fu *et al.*, 2007; Travella *et al.*, 2006; Vance and Vaucheret, 2001). In VIGS, viruses are engineered to carry host-derived sequences; the recombinant RNA sequences produced activate the RNA-mediated antiviral defense mechanism and direct the targeted degradation of endogenous mRNA sequences (Holzberg *et al.*, 2002; Lu *et al.*, 2003b). Because VIGS is a rapid, transient knockdown assay, it allows for the study of otherwise lethal phenotypes (Bruun-Rasmussen *et al.*, 2007). It does not require transformation or full-length cDNA sequences, making it an attractive method of gene silencing in complex cereals. VIGS is particularly useful in studying the homeologous loci of allopolyploid crops like wheat, because silencing is homology-dependent and transcripts sharing at least 85% sequence homology are also likely to be targeted for degradation (Scofield *et al.*, 2005; Scofield and Nelson, 2009).

The utility of VIGS in cereals

Although initial VIGS experiments were performed in dicots (Burch-Smith *et al.*, 2004), VIGS has seen rapid deployment in monocot species with the development of barley stripe mosaic virus (BSMV) as a suitable vector. BSMV is infectious in barley, wheat, oat and maize and is an appropriate vector for these crops (Robertson, 2004). It has recently been successfully used in gene silencing experiments in ginger, a more distantly related monocot (Renner *et al.*, 2009). Several studies using VIGS to investigate wheat–pathogen interactions have been reported (Cloutier *et al.*, 2007; Scofield *et al.*, 2005; Zhou *et al.*, 2007), but no experiments have been described where silenced wheat plants

were challenged with an insect pest. This led us to pursue VIGS as a way to investigate the role of two candidate genes, a *WRKY53* transcription factor and an inducible phenylalanine ammonia-lyase (*PAL*), both of which were differentially up-regulated during the wheat resistance response to *D. noxia* infestation (Botha *et al.*, 2010; Lapitan *et al.*, 2008).

WRKY transcription factors have a role in plant defense

The *WRKY* genes are a large family of plant-specific transcription factors with well-described roles in development and resistance to biotic and abiotic stresses (Eulgem, 2006). More than 70 *WRKYs* were found in *Arabidopsis*, and more than 100 were identified in rice. Their functions in regulating plant responses to microbial infection are only now becoming clear. *WRKYs* can act as positive or negative regulators of plant resistance against pathogens (Oh *et al.*, 2008), and can facilitate cross-talk between the antagonistic pathways regulated by jasmonate (JA) and salicylate (SA). *TaWRKY53* was recently identified as a gene differentially regulated upon *D. noxia* infestation in a resistant wheat line (Botha *et al.*, 2010). Differential up-regulation of *WRKY* genes in aphid-resistant plants has also been observed in sorghum (Park *et al.*, 2006), soybean (Li *et al.*, 2008), tobacco (Voelckel *et al.*, 2004) and wheat (Smith *et al.*, 2010). *TaWRKY53* is a group I *WRKY* transcription factor with two *WRKY* domains. Two homeologous cDNAs sharing 94% amino acid sequence identity, *TaWRKY53-a* and *TaWRKY53-b*, have so far been identified (Wu *et al.*, 2008). Because wheat is hexaploid, the existence of a third homeolog is presumed.

TaWRKY53 and its orthologs

Some understanding of the function of *TaWRKY53* has been gained from studies conducted on the orthologs *AtWRKY33* in *Arabidopsis* and *OsWRKY53* in rice. *AtWRKY33* is up-regulated by chitin-sensitive MAPK signaling pathways (Wan *et al.*,

2004), connecting MAPK cascades and downstream resistance effector genes (Qiu *et al.*, 2008). *AtWRKY33* is induced by oxidative stress and SA (Lippok *et al.*, 2007) and affords resistance against necrotrophic fungal pathogens (Zheng *et al.*, 2006), as well as increased salt tolerance (Jiang and Deyholos, 2009). *AtWRKY33* is a positive regulator of JA- and ET-mediated defense signalling but is a negative regulator of SA-mediated responses; the pathogen-induced expression of *AtWRKY33* occurs independently of SA (Lippok *et al.*, 2007; Zheng *et al.*, 2006). This may be of significance because aphid feeding in cereals induces both JA- and SA-signalling pathways (Zhao *et al.*, 2009). Like *AtWRKY33*, *OsWRKY53* in rice is induced by chitin oligosaccharides and stimulates the expression of PR proteins and peroxidase enzymes (Chujo *et al.*, 2007). The promoter regions of both *OsWRKY53* and *AtWRKY33* contain W-box elements, which are targets for either self-regulation or other regulatory *WRKYs* and enhance their pathogen-specific activity (Chujo *et al.*, 2009; Lippok *et al.*, 2007).

PAL activity is important in aphid defense

The enzyme phenylalanine ammonia-lyase catalyzes the deamination of L-phenylalanine to produce trans-cinnamate, a substrate for the synthesis of several plant secondary metabolites in the phenylpropanoid pathway. *PAL* genes are induced by incompatible interactions or pathogen-derived elicitors and are instrumental in plant defense against microbial infection. Exactly how *PAL* expression contributes to resistance has been the focus of much research, because it is involved in the synthesis of phytoalexins, which are directly antimicrobial, as well as in structural reinforcement via lignification and the production of SA (Dixon *et al.*, 2002; Mauch-Mani and Slusarenko, 1996). SA is a well-described regulator of plant defense mechanisms, vital to coordinating the oxidative burst and the generation of systemic acquired resistance; its synthesis is under the indirect control of *WRKY* transcription factor networks (Metraux, 2002), which also target phenylpropanoid-biosynthetic genes. The role of the phenylpropanoid pathway in

pathogen defense has been investigated in cereal crops: *PAL* is up-regulated in wheat cultivars resistant to *Fusarium graminearum* (Golkari *et al.*, 2009), and transgenic wheat engineered to express the *AMP1* antimicrobial gene from onion exhibits increased expression of *PAL* and elevated levels of SA upon challenge with a fungal pathogen (Subhankar *et al.*, 2006). Aphid infestation has also been demonstrated to induce *PAL* in resistant lines of barley (Chaman *et al.*, 2003) and wheat (Han *et al.*, 2009; Smith and Boyko, 2007).

The functions of *WRKY53* and *PAL* in aphid resistance are unknown. To ascertain their requirement for resistance in a genetically resistant wheat line, we investigated the effect of knocking down *WRKY53* and *PAL* transcription in resistant wheat on plant defense and aphid fitness using VIGS.

MATERIALS AND METHODS

Plant material

All experiments were conducted using hexaploid wheat (*Triticum aestivum* L.) cv. 'Gamtoos' (GS) and a resistant near-isogenic line 'Gamtoos-R' (GR), which was derived from successive backcrossing of wheat accession 94M370, containing the *Dn7*-bearing 1RS/1BL translocation from rye (*Secale cereale* L.) to the susceptible cultivar 'Gamtoos' (GS) (Anderson *et al.*, 2003; Marais *et al.*, 1994). All viral inoculations were performed on GR material, and uninoculated GR and GS plants served as resistant and susceptible controls, respectively. Each of the five treatment groups consisted of 12 plants, grown under greenhouse conditions as detailed by Lapitan *et al.* (2007). Pots were covered with nylon screen cloth to prevent premature aphid infestation prior to viral inoculation.

Samples from plants were not pooled, and each plant was observed as an independent biological replicate for a total of 12 biological replicates per treatment.

Silencing construct development

PCR products used in the construction of silencing vectors were amplified from wheat cDNA using the VIGS primers listed in Table 2.2. Primers were designed using PrimerQuest (Integrated DNA Technologies, Coralville, IA, USA) and used to amplify a 275-bp fragment of an inducible *PAL* transcript (accession AY005474) and a 338-bp fragment of Ta*WRKY53-a* (accession EF368357). PCR products were cloned into the pGEM-T Easy Vector (Promega, Madison, WI, USA) and excised using *Not*I (New England Biolabs, Ipswich, MA, USA), thereby generating *Not*I ends. These fragments were subsequently cloned into the *Not*I site of pSL038-1, a plasmid encoding a modified BSMV *γ* genome segment with a cloning site downstream of the *γ*b gene (Cakir and Scofield, 2008). The orientation of the cloned inserts was determined via PCR using a combination of vector-specific (Cakir and Scofield, 2008) and fragment-specific primers (Table 2.2). Clones putatively containing the fragments in the antisense orientation relative to the *γ* genes were sequenced to confirm their identity and subsequently used for gene silencing.

Viral inoculation and aphid infestation

The *α*, *β* and *γ* RNAs of the BSMV genome were synthesized from linearized plasmids containing cloned cDNA genome segments (Petty *et al.*, 1989), using the mMessage mMachine T7 kit (Ambion, Austin, TX, USA). Capped *in vitro* transcripts of each RNA segment were combined in an equimolar ratio and added to an abrasive FES buffer (0.1 M glycine, 0.06 M K2HPO4, 1% w/v tetrasodiumtetrasodium pyrophosphate, 1% w/v bentonite, 1% w/v celite, pH 8.5) according to the procedures of Scofield et al. (2005). BSMV$_{WRKY53}$ was constituted from *α*, *β* and *γ* with the *WRKY53-a* insert; BSMV$_{PAL}$ was

constituted from α, β and γ with the *PAL* insert; BSMV$_o$, which served as viral control, was constituted from α, β and γ RNA derived from the original empty pSL038-1 vector. A volume corresponding to 3 μg of viral RNA was rub-inoculated onto the first and second leaves of GR seedlings at the 3–4 leaf stage. Plants were mass-infested with ~50 Russian wheat aphid biotype RWA2 apterous adults 5 days after viral inoculation, and pots were again covered in nylon screen cloth to prevent aphids from escaping.

Quantitative PCR

Six days after aphid infestation (11 days after viral inoculation), the distal 12 cm of third leaves from four experimental plants per treatment was collected into liquid nitrogen. RNA was extracted from each individual leaf sample with the Trizol reagent (Invitrogen, Carlsbad, CA, USA). Each sample was subjected to DNase I treatment (Promega, Madison, WI, USA) and purified using RNeasy spin columns (Qiagen, Hilden, Germany). First-strand cDNA synthesis was conducted using the Retroscript reagent (Ambion) and primed with random decamers. All real-time quantitative PCR (RT-qPCR) amplifications were performed on the iCycler iQ instrument (Biorad, Hercules, CA, USA) using the Perfecta SYBR Green Supermix (Quanta Biosciences, Gaithersburg, MD, USA) and input cDNA equivalent to 2.5 ng of total RNA. The following cycling parameters were used: initial denaturation at 95 °C for 2 min; 50 amplification cycles consisting of denaturation at 95 °C for 15 s, annealing and extension at 57 °C for 45 s. Single-fragment amplification was verified by dissociation curve analysis. Gene expression values were standardized across four independent biological replicates, with each sample amplified in triplicate (Willems *et al.*, 2008). Relative transcript abundance was calibrated to the mean expression of the GR treatment group and normalized against the level of 18S rRNA in each sample (Pfaffl, 2001).

DAB staining for H_2O2

DAB staining was performed according to the protocol of Thordal-Christensen *et al.* (1997). Fourth leaves from four independent biological replicates per treatment were collected 6 days after aphid infestation (11 days after viral inoculation). Feeding aphids were removed from all leaves with a paintbrush, and leaves were placed in 1 mg mL^{-1} 3,3'-diaminobenzidine (DAB)-HCl, pH 3.8, (Sigma-Aldrich, St. Louis, MO, USA) and incubated overnight in the dark at room temperature with gentle agitation. The tissues were subsequently cleared in 75% ethanol at 37 °C with gentle agitation for 5 h, replacing the ethanol as needed. The presence of H_2O_2 is revealed by reddish-brown polymerized deposits.

Aphid reproduction measurements

Four days after viral inoculation, single clip cages were attached to the third leaves of all experimental plants including uninoculated controls. Two adult, apterous aphids were placed inside each clip cage, and all aphids but one newborn nymph were removed the following day. This nymph constituted the foundress, and its reproduction was monitored during the extent of the experiment; this varied according to the intrinsic rate of increase (r_m) for each individual foundress. Because some foundresses were lost because of handling, intrinsic rate of increase was estimated for ten aphids per treatment, according to the equation $r_m = (0.738 \times \ln(M_d)) / d$ developed by Wyatt and White (1977), where d is the number of days comprising the prenymphipositional period and M_d is the number of offspring produced in the period from d to $2d$ days after production of the first nymph.

RESULTS

Silencing reduces transcript abundance

Recombinant silencing vectors were generated from wheat cDNA fragments corresponding to the *WRKY53* and *PAL* genes previously identified as induced during *D. noxia* resistance responses. Silencing was performed on a resistant near-isogenic line, 'Gamtoos-R' (GR). Eleven days after viral inoculation of GR plants and 6 days after *D. noxia* infestation of silenced plants and controls, leaf tissue was collected to determine the efficiency of silencing. Real-time quantitative PCR (RT-qPCR) was performed to determine changes in *WRKY53* and *PAL* transcript abundance in each treatment group (Figure 2.1). The GR plants inoculated with the BSMV$_{WRKY53}$ construct and infested with aphids showed a 78.87% mean reduction in *WRKY53* transcript accumulation compared to uninoculated, aphid-infested GR controls ($P < 0.0001$) (Figure 2.1a). GR+BSMV$_o$ viral controls were not statistically different from resistant GR controls ($P = 0.1550$). *WRKY53* transcript levels in GR+BSMV$_{WRKY53}$ plants were similar to those measured in susceptible GS controls ($P = 0.4374$). Mean *WRKY53* levels in GR plants inoculated with the BSMV$_{PAL}$ construct were not significantly different from GR controls ($P = 0.8566$). The GR+BSMV$_{PAL}$ plants showed a 59.25% mean reduction in *PAL* transcript accumulation compared to GR control plants ($P < 0.0001$) (Figure 2.1b); transcript levels were similar to that of GS controls ($P = 0.7836$). Surprisingly, mean *PAL* levels in GR+BSMV$_{WRKY53}$ plants were also suppressed to levels similar to the GS controls ($P = 0.1518$).

Silencing induces susceptibility to D. noxia

Gene silencing phenotypes generated via VIGS were highly variable, not only between different plants inoculated with the same vector construct, but also between leaves of a single experimental plant. Only GR and GR+BSMV$_o$ plants developed clear hypersensitive lesions typical of a *Dn7*-mediated aphid resistance response (Figure

2.2a,b). Viral streaking in GR+BSMV$_o$ inoculation control plants (Figure 2.2b) and altered phenotypes in silenced plants were most discernable in emerging leaves and tillers. This agrees with previous observations that BSMV is more readily amplified in emerging phloem sink tissues (Holzberg *et al.*, 2002). Chlorosis and leaf rolling, classic symptoms of susceptibility to *D. noxia* (Burd and Burton, 1992), were observed in both GR+BSMV$_{PAL}$ and GR+BSMV$_{WRKY53}$ treatment groups (Figure 2.2c,d). Although the GR+BSMV$_{PAL}$ treatment group exhibited the most severe bleaching, the GR+BSMV$_{WRKY53}$ plants succumbed slightly earlier to aphid infestation. Sink tissues of both silenced groups most closely resembled the GS plants (Figure 2.2e).

Silencing alters the oxidative burst

To investigate possible alterations to the functioning of the oxidative burst in GR plants when *WRKY53* or *PAL* transcripts are silenced, leaves were collected and stained for H_2O_2 using 3,3'-diaminobenzidine (DAB), which forms reddish-brown polymerized deposits in the presence of peroxidase (Figure 2.3). Dark staining was observed in all GR and GR+BSMV$_o$ samples around the feeding sites (Figure 2.3a,b), but staining was slight in all GS, GR+BSMV$_{PAL}$ and GR+BSMV$_{WRKY53}$ samples (Figure 2.3c,d,e). However, cut ends of all leaf samples showed similar levels of dark polymerized DAB deposits (not shown). This indicates that only the generation of aphid-induced reactive oxygen species (ROS) is impeded and not the generation of ROS owing to wounding.

Aphid performance improves on silenced plants

To ascertain whether *WRKY53* or *PAL* contributes to the antibiotic effect of *Dn7*-mediated resistance in GR plants, individual aphids were caged on silenced plants and their reproductive capacity was monitored. Rate of reproduction is an easily calculated component of absolute fitness (Orr, 2009), and antibiosis is defined as a category of aphid resistance that negatively impacts aphid reproduction (Smith *et al.*, 1992). Mean

total number of nymphs were calculated for aphids feeding on different silenced and control plants as a measure of fertility (Figure 2.4). After 16 days of feeding on susceptible GS plants, aphids had a mean total of 70 offspring, while aphids feeding on GR had a mean total of 14 only offspring. By contrast, aphids feeding on GR+BSMV$_{PAL}$ and GR+BSMV$_{WRKY53}$ plants had 21 and 29 mean total offspring, respectively. Intrinsic rate of increase (r_m) was calculated as a measure of fecundity for aphids caged on different treatment groups (Table 2.1). Aphids feeding on GS plants had the highest calculated rate of increase (r_m = 0.233, s = 0.020) and aphids feeding on GR plants had the lowest rate of increase (r_m = 0.089, s = 0.016), which was not significantly different from that calculated for aphids feeding on GR+BSMV$_o$ viral inoculation controls. Silencing resulted in significantly higher rates of increase for both GR+BSMV$_{PAL}$ (r_m = 0.128, s = 0.018) and GR+BSMV$_{WRKY53}$ (r_m = 0.147, s = 0.016) when compared to GR (P < 0.0001). Silencing of *WRKY53* had a significantly greater effect on intrinsic increase rate than silencing *PAL* (P = 0.0313), which might be indicative of its potential regulatory role in aphid resistance networks. Taken together, data gathered on aphid reproduction indicate silencing either *WRKY53* or *PAL* interferes with the plant's ability to successfully initiate an antibiotic defense response.

DISCUSSION

Our study tested the requirement for the *WRKY53* transcription factor and an inducible *PAL* gene during a successful defense response against *D. noxia* infestation in wheat. These genes were selected based on dissimilar patterns of gene expression exhibited between resistant and susceptible near-isogenic wheat lines upon *D. noxia* infestation (Botha *et al.*, 2010; Lapitan *et al.*, 2008). Using recombinant BSMV vectors, we induced targeted posttranscriptional gene silencing of these candidate genes in resistant wheat,

leading to chlorosis and leaf rolling, as well as enhancing the fecundity of aphids feeding on silenced plants.

The levels of silencing (about 59% for *PAL* and 79% for *WRKY53*) achieved in this study are comparable to those reported by others using VIGS in wheat: the popular VIGS marker gene phytoene desaturase (*PDS*) is routinely silenced by 60% compared to controls (Bruun-Rasmussen *et al.*, 2007; Scofield *et al.*, 2005), and the expression of pathogen defense genes *RAR1* and *SGT1* could be reduced by 54% and 83%, respectively (Scofield *et al.*, 2005). We were able to knock down transcript abundance of *PAL* and *WRKY53* in GR plants to levels similar to those measured for GS plants. The resulting phenotypes were indistinguishable from the susceptible phenotype of GS, with plants in both silencing treatments exhibiting leaf rolling and chlorosis. Leaf rolling is thought to result from feeding-induced osmotic dysregulation (Burd and Burton, 1992) and chlorosis from oxidative damage to the photosynthetic machinery (Botha *et al.*, 2006; Heng-Moss *et al.*, 2003). This suggests that the phenylpropanoid pathway might play a role in preventing both kinds of symptoms, perhaps coordinately regulated via large networks of WRKY and other transcription factors.

Abrogating the function of *WRKY53* had a more adverse effect on the antibiotic capacity of GR plants than knocking down *PAL* and also resulted in reduction in *PAL* transcripts. This indicates that *WRKY53* likely modulates the expression of a variety of defense-related genes, of which *PAL* and the phenylpropanoid pathway may be a subset. Whitefly infestation in *Arabidopsis* induces both phenylpropanoid synthetic genes and *AtWRKY70* (Kempema *et al.*, 2007), and the promoter region of the *Arabidopsis* phenylpropanoid gene *At4Cl4* has three W-boxes, two of which have demonstrated WRKY binding activity (Hahlbrock *et al.*, 2003). The attenuation effect observed via silencing is likely attributable to an indirect regulatory mechanism, although the

potential for the wheat WRKY53 protein to directly interact with the promoter region of the *PAL* gene should be investigated.

The mode of resistance rendered by the *Dn7* gene has been characterized as antibiosis (Lazzari *et al.*, 2009), affording high levels of resistance to several different *D. noxia* biotypes (Randolph *et al.*, 2009). Both *WRKY53* and *PAL* presumably function downstream of *Dn7* in a defense gene cascade, leading to the production of salicylic acid, an important regulator of the oxidative burst, and also the production of defensive phytoalexins via the phenylpropanoid pathway. Silencing of either gene in our study disrupted the *Dn7*-mediated production of H_2O_2. Interestingly, the *Dn1* gene initiates a rapid oxidative burst during *D. noxia* feeding and also specifically confers antibiosis (Van der Westhuizen *et al.*, 1998). Lowered levels of ROS coupled with increased rates of aphid reproduction in silenced plants provide further evidence that the oxidative burst forms an integral component of antibiotic resistance. Whether the oxidative burst facilitates antibiosis only through defense signal transduction mediated by SA, or additionally through direct damage to the aphid through ingestion of free radicals (Botha *et al.*, 2005), still needs to be determined.

Previous reports on the use of VIGS in cereals noted the heterogeneity of silencing phenotypes (Bruun-Rasmussen *et al.*, 2007; Scofield *et al.*, 2005). We expected and observed variability within treatments among plants and also between tissues of the same plant at different developmental stages. The efficacy of silencing may decline rapidly over time, and this may depend on both the insert size of the VIGS construct and the nature of the targeted sequence (Bruun-Rasmussen *et al.*, 2007). Visual phenotypes may take several days to develop after viral inoculation and, because VIGS is a transient assay, may decline before the plants reach maturity. Because the generation time of *D. noxia* can vary from 8 to 42 days (Aalbersberg *et al.*, 1987), the experimental window during which optimal silencing and aphid reproduction overlap might be small. Caution

should therefore be exercised when planning VIGS-based aphid feeding experiments: the timing of viral inoculation and aphid infestation is critical.

This is the first report of VIGS being successfully used to target and silence genes thought to be involved in wheat resistance against aphids. The usual symptoms of BSMV infection, such as viral streaking, did not mask the effects of gene knockdown and did not influence aphid behavior or expression of target genes to any great extent. Similarly, when VIGS was used to study the function of the *Lr1* and *Lr21* rust resistance genes, the presence of virus did not confound results (Cloutier *et al.*, 2007; Scofield *et al.*, 2005). VIGS therefore has the potential to become a very useful and routine approach to validating candidate gene function in plant–insect interactions. It is of particular benefit to research in polyploid species recalcitrant to conventional transformation techniques, such as wheat. The VIGS system can potentially be scaled up for high throughput screening of hundreds of candidate genes in a short period of time (Lu *et al.*, 2003a). Moreover, because silencing of more than one target transcript was recently demonstrated with double vectors (Cakir and Scofield, 2008), VIGS can be employed to study epistatic interactions between genes. This attribute of the system could be exploited as aphid defense signal transduction networks are mapped out. Successful use of VIGS in conjunction with pest and pathogen resistance assays constitutes a major advance in interpreting the profusion of wheat stress transcriptomics information currently available. Candidate genes verified to be directly responsible for resistant phenotypes will be of tremendous interest to programs breeding for durable pest resistance.

Table 2.1. Aphid fecundity estimated using intrinsic rates of increase

Treatment	r_m	*s*
GR	0.089*	0.016
GR+BSMV$_0$	0.093*	0.024
GR+BSMV$_{PAL}$	0.128	0.018
GR+BSMV$_{WRKY53}$	0.147	0.016
GS	0.233	0.020

r_m, intrinsic rate of increase; *s*, standard deviation; GR, 'Gamtoos-R' resistant NIL (*Dn7*); GS, 'Gamtoos' susceptible cultivar; BSMV$_0$, viral inoculation control; BSMV$_{PAL}$, *PAL*-silencing vector; BSMV$_{WRKY53}$, WRKY53-silencing vector; *n* = 10 aphids per treatment.
*Not significantly different (*P* = 0.659).

Table 2.2. Wheat primers

Accession	Type	Target	Primer Sequence
AY005474	VIGS	34-308	5'-AAGCTGCTCAACGCCAATGTCA-3'
			5'-TCAAAGAGCACGGTCGATGCAA-3'
	qPCR	745-848	5'-AAGCTGATGTTCGCGCAGTTCT-3'
			5'-AAACCATAGTCCAAGCTCGGGT-3'
EF368357	VIGS	1083-1420	5'-GTTGTCAAGGGCAATCCCAACC-3'
			5'-TCGTCCTTGGTGCGCTGGAA-3'
	qPCR	36-139	5'-TCGATCGCCATGTCCTCCTTCA-3'
			5'-CCAGACCCTGATAGAAGCTCAGTCAA-3'

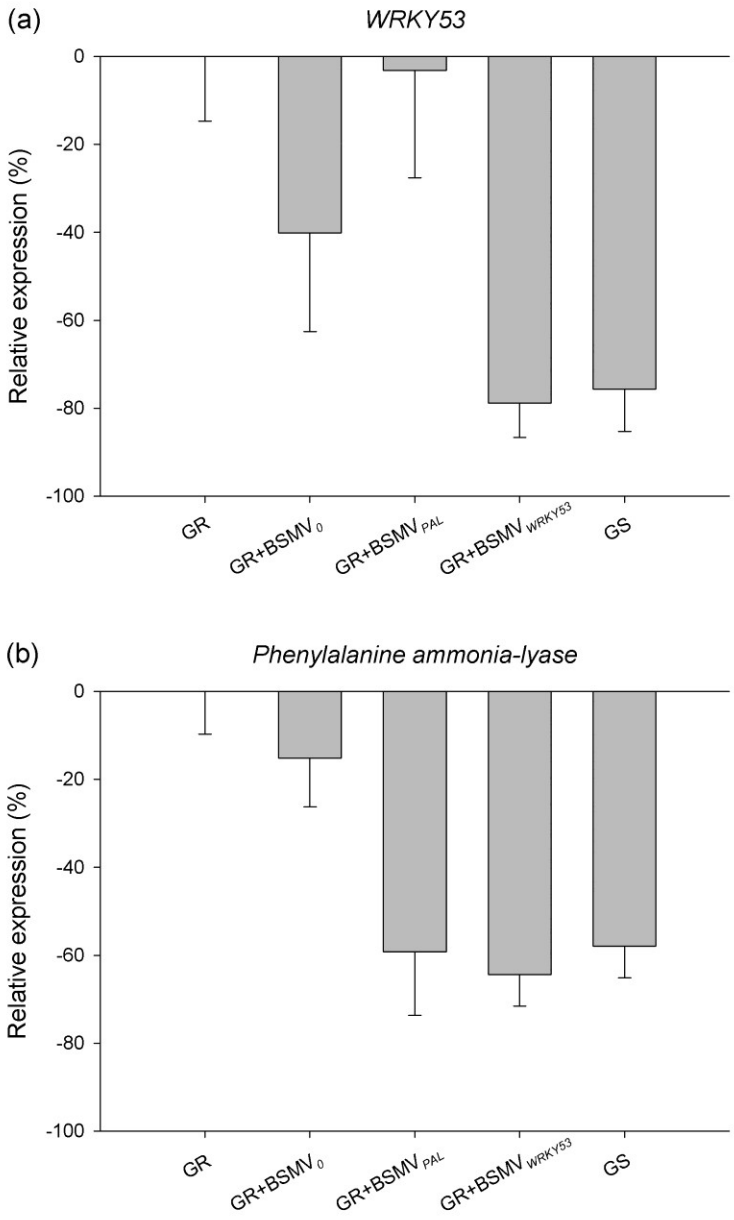

Figure 2.1. Silencing efficiency as revealed by RT-qPCR. Gene expression values were standardized across four independent biological replicates, with each sample amplified in triplicate. (a) Expression of *WRKY53* calibrated to the mean level of expression in the GR treatment. (b) Expression of the inducible *PAL* gene calibrated to the mean level of expression in the GR treatment.

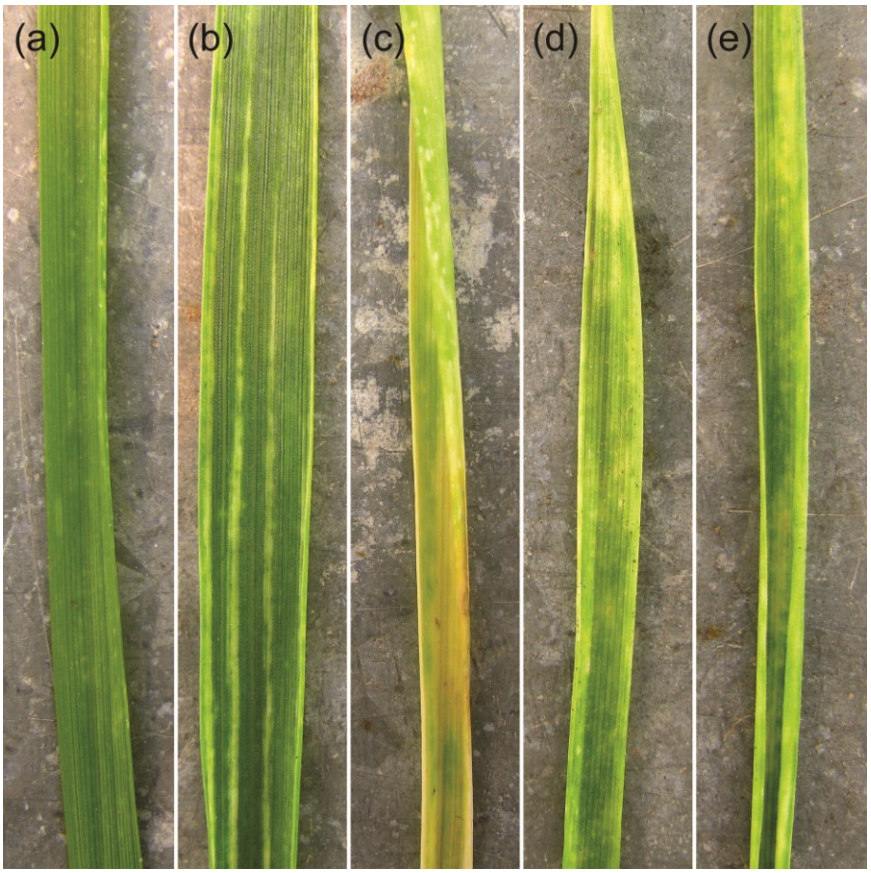

Figure 2.2. Phenotypes of leaves from silenced plants and controls 18 days after aphid infestation. (a) Uninoculated GR, exhibiting necrotic spots indicative of a hypersensitive response. (b) GR+BSMV$_{\gamma}$ exhibiting longitudinal streaking indicative of viral infection. (c) GR+BSMV$_{PAL}$ showing leaf rolling and chlorosis. (d) GR+BSMV$_{WRKY53}$ showing leaf rolling and chlorosis. (e) GS exhibiting typical symptoms of aphid susceptibility, including leaf rolling and chlorosis. Leaves are representative of at least 10 plants per treatment.

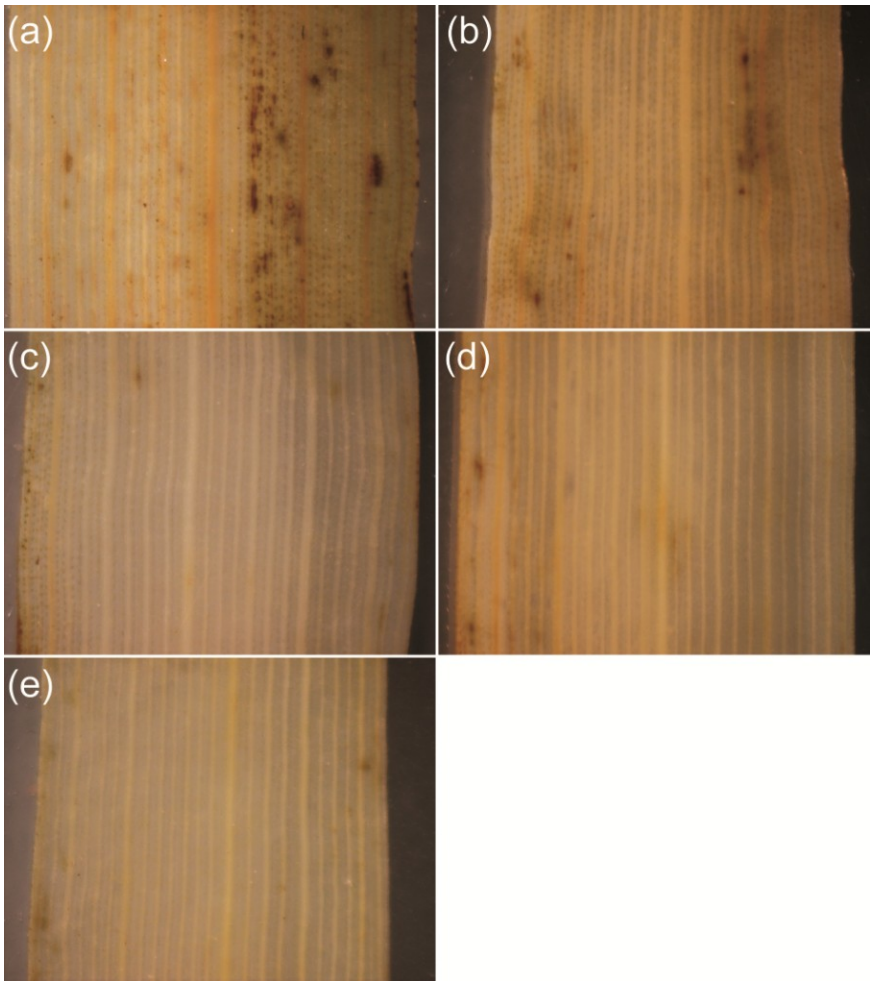

Figure 2.3. Silencing of *PAL* and *WRKY53* results in reduced peroxide accumulation at aphid feeding sites. Leaves of silenced and control plants were stained with 3,3'-diaminobenzidine (DAB) 6 days after aphid feeding. Leaves are representative of four independent biological replicates per treatment. (a) and (b) GR and GR+BSMV$_o$, respectively, both tissues showing dark areas around aphid feeding sites indicating the presence of peroxide. (c), (d) and (e) GR+BSMV$_{PAL}$, GR+BSMV$_{WRKY53}$ and GS, respectively, showing much reduced H_2O_2 staining.

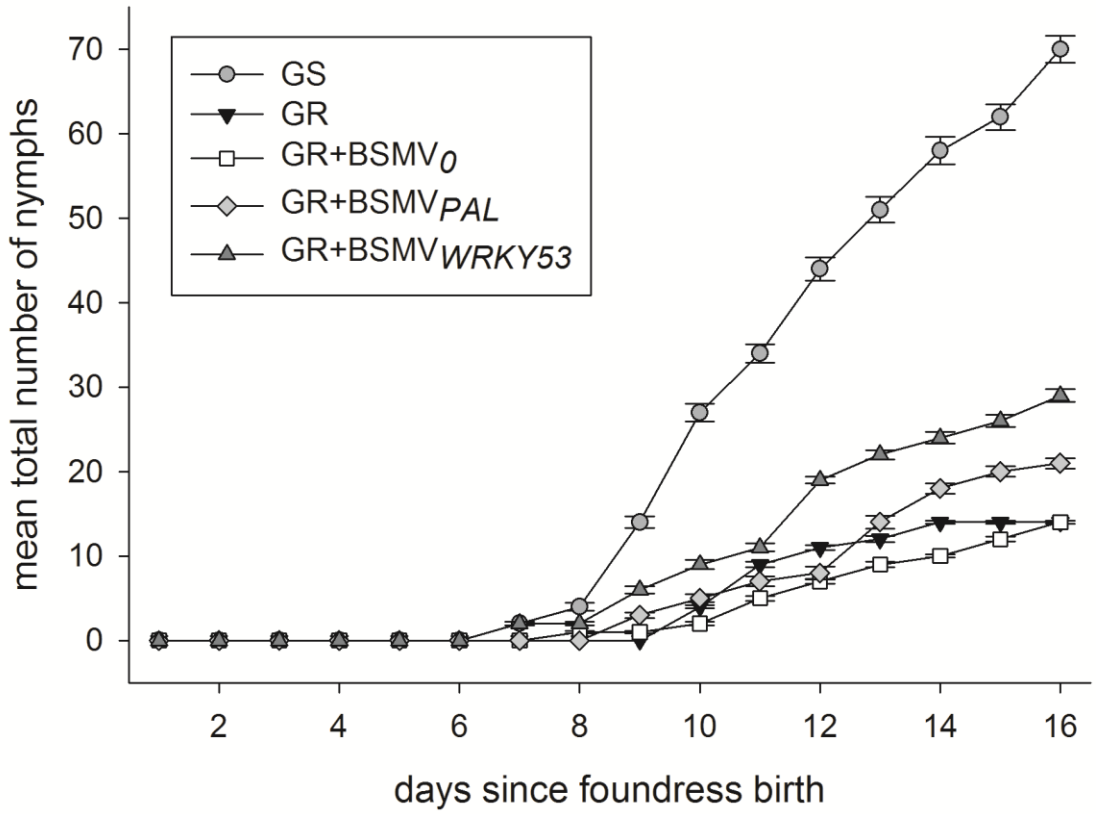

Figure 2.4. Mean total nymph production of 10 aphids per treatment, independently assayed over 16 days. Aphids feeding on GS and GR had the highest and lowest mean number of offspring, respectively.

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CHAPTER 3:

CHARACTERIZATION OF THE *WRKY53* TRANSCRIPTIONAL NETWORK

SUMMARY

The transcription factor WRKY53 is induced by a wide array of biotic and abiotic stresses in cereals, and is an essential component of the aphid resistance response of wheat. However, very little is known about the structure, regulation and function of the wheat ortholog. Our objective was to more thoroughly characterize the genomic region encoding the wheat *WRKY53* gene and its *cis*-acting regulatory elements, and to determine other genetic components of the WRKY53 transcriptional network, including regulators upstream from *WRKY53* in the defense signal cascade as well as downstream target genes. We used a PCR-based approach to obtain the 1.2 kb promoter region as well as a full-length genomic clone of the wheat *WRKY53* gene. The gene is composed of five exons, similar to the rice ortholog. The promoter region of wheat WRKY53 has three W-box WRKY-binding domains, consistent with the distribution of W-boxes in orthologs from other species. Sequence analysis of WRKY53 alleles from different Asian landraces with different levels of aphid resistance found that evolution of the gene is largely functionally constrained, with very few amino acid substitutions between accessions. Based on the few substitutions that were observed, especially in the transactivating N-terminal region of the protein, the accessions could be grouped according to aphid resistance, as opposed to geographic center of origin. Based on gene coexpression

networks in rice and the presence of W-boxes in their 1 kb promoter regions, we predicted a chitinase, a peroxidase and a receptor kinase to be likely downstream targets of *WRKY53*, and tested the ability of recombinant WRKY53 protein to interact with their promoter regions using an electrophoretic mobility shift assay. To discover novel protein-protein interactions involving WRKY53, we performed yeast two-hybrid analyses using a rice cDNA library enriched for pathogen-responsive genes. A microsomal glutathione *S*-transferase showed a strong interaction with the WRKY53 protein, offering further support for the regulatory role of WRKY53 during the oxidative burst. A yeast one-hybrid approach was used to discover novel proteins able to interact with the promoter region of WRKY53 and modulate its function. We discovered a calmodulin-related calcium sensor protein, an ultraviolet-B repressible protein and a DUF584 protein as possible upstream regulators of the WRKY53 network. Collectively, our data suggest that TaWRKY53 acts as a transcriptional regulator of several defense-related pathways in wheat.

INTRODUCTION

The WRKY gene family: structure and function

In 1994, Japanese researchers reported the discovery of a novel DNA-binding protein in sweet potato, which they called Sweet Potato Factor 1, or SPF1 (Ishiguro and Nakamura, 1994). However, it was only two years later that a rival group at the Max Planck Institute for Plant Breeding Research demonstrated that specific pathogen response elements in the promoters of *PR* genes in parsley were bound by a whole family of these proteins, which were dubbed WRKY proteins after the unique conserved amino acid sequence of the DNA-binding domain (Rushton *et al.*, 1996). *WRKY* genes are not exclusive to plants, with a single *WRKY* gene also found in the green alga *Chlamydomonas*

reinhardtii (Zhang and Wang, 2005), the slime mold *Dictyostelium discoideum* (Glockner *et al.*, 2002) and the diplomonad *Giardia lamblia* (Pan *et al.*, 2009). However, *WRKY* genes have proliferated in plant genomes to become one of the largest plant transcription factor families (Eulgem, 2005; Ulker and Somssich, 2004). *WRKY* genes are well-distributed across plant chromosomes (Eulgem *et al.*, 2000), but tend to cluster in duplicated regions of the genome. Gene expression analysis suggests that tandemly duplicated *WRKYs* have rapidly divergent expression patterns (Ramamoorthy *et al.*, 2008), although at least some *WRKYs* are functionally redundant (Lippok *et al.*, 2007). *WRKYs* can act as positive and negative regulators of gene expression (Oh *et al.*, 2008; Rushton *et al.*, 2010) and have a regulatory role in a wide range of processes, including senescence (Miao *et al.*, 2004), abiotic stresses such as wounding (Chen *et al.*, 2010), temperature extremes (Li *et al.*, 2011; Wu *et al.*, 2009) and salinity (Jiang and Deyholos, 2009), and also responses to attack by biotic stressors such as viruses (Liu *et al.*, 2004), bacterial and fungal pathogens (Berri *et al.*, 2009; Mangelsen *et al.*, 2008; Murray *et al.*, 2007) and insect pests (Izaguirre *et al.*, 2003; Park *et al.*, 2006).

The DNA binding site targeted by WRKY proteins is the W-box of consensus sequence (C/T)TGAC(C/T) (Eulgem and Somssich, 2007), although neighboring nucleotides contribute to binding affinity and specificity (Ciolkowski *et al.*, 2008). Based on the characteristics of their conserved WRKY domains and zinc finger motif, WRKY transcription factors can be classified into distinct groups. Group I proteins feature two WRKY domains, while Group II and III only exhibit a single WRKY domain. Group I and II have a Cys_2–His_2-type zinc finger motif, whereas Group III has a Cys_2–His–Cys zinc finger (Rushton *et al.*, 2010). However, Group II is not monophyletic and based on phylogenetic evidence, can be further divided into five subgroups (Zhang and Wang, 2005).

WRKY53 is a stress-responsive WRKY gene in cereals

There are a total of 103 *WRKY* genes in the rice (*Oryza sativa*) genome, distributed across all chromosomes (Ramamoorthy *et al.*, 2008). To date, the only attempt at a systematic census of *WRKY* transcription factors in wheat (*Triticum aestivum*) has been conducted by Wu and colleagues (2008), who isolated 15 wheat cDNAs encoding *WRKY* genes and documented their differential expression during plant development and under various environmental stresses. The wheat *WRKYs* are assigned gene names based on nucleotide sequence similarity to their rice orthologs. Therefore, *TaWRKY53*, the focus of this study, is the wheat ortholog of *OsWRKY53* in rice. In fact, two distinct but highly related *TaWRKY53* cDNAs have been reported, designated *TaWRKY53-a* (accession EF368357) and *TaWRKY53-b* (accession EF368364). This is not surprising, since wheat is a hexaploid exhibiting homeologous loci across its three genomes; the existence of a third homeolog of *TaWRKY53* is therefore likely. Given the high degree of sequence similarity between *TaWRKy53-a* and *TaWRKY53-b,* they are anticipated to have significant functional overlap, although they may not necessarily be functionally redundant. Although very little is known about the function of *TaWRKY53*, especially in the context of plant defense against biological stress, much can be gleaned from research on orthologous loci in model crop species.

The rice ortholog, *OsWRKY53*, was first identified from a cDNA microarray analysis performed on rice cell suspension cultures induced with chitinous oligosaccharide elicitors (Akimoto-Tomiyama *et al.*, 2003). Its genomic sequence was extracted from public genome databases and bioinformatically annotated using a rice-specific Hidden Markov Model (Xie *et al.*, 2005; Zhang *et al.*, 2004). OsWRKY53 is a group I WRKY transcription factor with two WRKY domains and a Cys_2–His_2 zinc finger motif. *OsWRKY53* is highly expressed in roots and leaves, and RT-PCR analysis found it to be upregulated during drought and salinity stress (Ramamoorthy *et al.*, 2008).

Overexpression of *OsWRKY53* greatly reduces symptoms of infection by the hemibiotrophic fungus *Magnaporthe grisea* (Chujo *et al.*, 2007).

AtWRKY33, the ortholog of *TaWRKY53* from *Arabidopsis thaliana*, is an important component of basal resistance and is rapidly induced by MAMPs (microbe-associated molecular patterns) such as flg22, the flagellin-derived peptide (Navarro *et al.*, 2004). It is upregulated in response to virulent and avirulent strains of *Pseudomonas syringae* pv. tomato DC3000 (Zheng *et al.*, 2006). *AtWRKY33* is induced by virulent and avirulent strains of biotrophic fungi (Lippok *et al.*, 2007), and overexpression leads to enhanced resistance to the necrotrophic fungi *Botrytis cinerea* and *Alternaria brassicicola* (Zheng *et al.*, 2006). *AtWRKY33* is also induced by oxidative stress (Mahalingam *et al.*, 2005) and constitutive overexpression of *AtWRKY33* enhances thermotolerance (Li *et al.*, 2011). It has recently been documented that *AtWRKY33* might also have a role in plant development, since overexpression leads to increased germination frequency, and T-DNA insertion mutants had reduced germination rates (Li *et al.*, 2011).

OsWRKY53/AtWRKY33 forms part of a MAP kinase signaling cascade

AtWRKY33 has been proposed as a positive regulator of jasmonic acid (JA) and ethylene (ET) dependent defense responses (Lippok *et al.*, 2007). Its possible role in salicylic acid (SA) mediated defense signaling is less clear, and the data reported from rice and *Arabidopsis* studies are conflicting. Zheng *et al.* (2006) reported that *Arabidopsis wrky33* T-DNA insertion mutants had enhanced PR protein expression, and *AtWRKY33* overexpressing plants had decreased PR protein expression. Conversely, Chujo et al. (2007) reported the induction of PR protein expression in *OsWRKy53* overexpressing rice plants. The fast induction of *AtWRKY33* signaling does not rely on *de novo* protein synthesis, but instead post-translational changes to a pre-existing pool of AtWRKY33

protein, most likely in the cytosol (Lippok *et al.*, 2007). This might reflect inherent differences between the rice and *Arabidopsis* orthologs. It has been hypothesized that the phosphorylation state of a WRKY protein might play an important part in whether it acts as repressor or activator (Turck *et al.*, 2004) and these conflicting studies did not investigate protein phosphorylation as an aspect of WRKY transcriptional control. Additionally, WRKY proteins are direct targets of MAP kinase signaling cascades. In *Arabidopsis*, AtWRKY33 is phosphorylated by the MAP kinases MPK3 and MPK6, which are rapidly induced by chitinous elicitors (Wan *et al.*, 2004) and are involved in the generation of reactive oxygen species (ROS) and hypersensitive plant cell death (Mao *et al.*, 2011). Interestingly, *AtWRKY33* expression is still induced by chitin in *mpk6* T-DNA insertion mutants (Wan *et al.*, 2004), hinting at either the functional redundancy between MPK3 and MPK6, or the MAP kinase-independent perception of chitin by AtWRKY33. The *Arabidopsis* response to chitin occurs independently of either JA/ET or SA-based signaling (Wan *et al.*, 2004), implying that there might be parallel WRKY-mediated pathways toward induction of defense responses.

Another MAP kinase, MPK4, appears to be a negative regulator of AtWRKY33, preventing the inappropriate activation of defense in the absence of attacking pathogens (Qiu *et al.*, 2008). In particular, it is thought that inactive AtWRKY33 is complexed to MPK4, together with a protein called MKS1 (Petersen *et al.*, 2010). Perception of pathogen attack initiates a MAP kinase signaling cascade: the MAP kinase kinase kinase MEKK1 actives the MKK1/MKK2 MAP kinase kinase module, which in turn activates MPK4. Activated MPK4 phosphorylates MKS1, and AtWRKY33 is released (Rushton *et al.*, 2010).

TaWRKY53 and aphid resistance in wheat

The significance of WRKY genes in cereal responses against aphid attack is only now becoming clear (Botha *et al.*, 2010; Smith *et al.*, 2010). Since we have found that *TaWRKY53* is not only differentially regulated during the wheat response to the Russian wheat aphid (*Diuraphis noxia* Kurdjumov), but required for the establishment of successful resistance (Van Eck *et al.*, 2010), we wanted to characterize this wheat gene and its regulatory role in more detail. Apart from some EST information, almost nothing is known about the structure and function of *TaWRKY53*. We therefore characterized the structure of the gene and its promoter region, before identifying upstream and downstream genetic components of the *TaWRKY53* transcriptional network.

MATERIALS AND METHODS

TaWRKY53 promoter isolation

Genome walking was performed using the GenomeWalker Universal Kit (Clontech, Mountain View, CA, USA) according to the manufacturer's instructions. Nested primers specific to the 5' end of the *TaWRKY53* coding sequence (CDS) were designed based on accession EF368357, a *WRKY53* cDNA clone isolated from hexaploid wheat (*Triticum aestivum* L.) cv. 'Nongda 3338' (Wu *et al.*, 2008). Genomic DNA isolated from cv. 'Gamtoos-R' (GR) was digested in four separate 2.5 µg reactions with 80 U of *Dra*I, *Eco*RV, *Pvu*II and *Stu*I blunt cutting restriction endonucleases, forming four digest libraries. Purified digests were ligated to the GenomeWalker adaptor and subjected to a primary PCR amplification reaction using an adaptor-specific primer, gene-specific primer TaWRKY53_GSP1 (Table 3.1) and LongAmp *Taq*, a high-fidelity DNA polymerase (New England Biolabs, Ipswich, MA, USA). The following cycling parameters were used: initial denaturation at 94 °C for 30 s; 7 amplification cycles consisting of

denaturation at 94 °C for 25 s, annealing and extension at 72 °C for 3 min; 32 amplification cycles consisting of denaturation at 94 °C for 25 s, annealing and extension at 67 °C for 3 min; final extension at 67 °C for 7 min. The presence of amplification products was verified by agarose gel electrophoresis. A 50× dilution of the primary PCR was subsequently used as template for a secondary PCR using a nested adaptor-specific primer and gene-specific primer TaWRKY53_GSP2 (Table 3.1). Individual amplicons were gel purified, cloned into the pGEM-T Easy vector (Promega, Madison, WI, USA) and sequenced.

Genome walking fragment assembly and characterization

Sequence reads obtained from genome walking were assembled into contigs using Geneious Pro 5.4 (Drummond *et al.*, 2011). Promoter characterization was performed using a combination of the *cis*-acting regulatory element databases PLACE (Higo *et al.*, 1999) and PlantCARE (Lescot *et al.*, 2002), and manual scans for the presence of putative W-boxes using the (C/T)TGAC(C/T) consensus sequence.

WRKY53 CDS and gene model

Primers TaWRKY53_CDS_fwd and TaWRKY53_CDS_rvs (Table 3.1) were used to amplify the CDS of *TaWRKY53* out of cDNA and genomic DNA from GR wheat. The following cycling parameters were used: initial denaturation at 94 °C for 1 min; 37 amplification cycles consisting of denaturation at 94 °C for 20 s, annealing at 60 °C for 20 s, extension at 65 °C for 1:40; final extension at 65 °C for 7 min. PCR products were cloned into the pGEM-T Easy vector and sequenced. Sequences were assembled and aligned with GenBank accessions EF368357 and EF368364 to confirm their identity.

WRKY53 allelic diversity assay

Genomic DNA was isolated from six genetically divergent Asian landraces of wheat that exhibited high diversity at 81 SSR loci (Peng *et al.*, 2009) and either resistance or

susceptibility to *D. noxia*. Two US varieties, 'Yuma' and 'Halt', both susceptible to *D. noxia* infestation, were also included (Table 3.2). Primers TaWRKY53_CDS_fwd and TaWRKY53_CDS_rvs (Table 3.1) were used to amplify the full-length CDS from all accessions. GR wheat was included as a previously-characterized control. All PCR products were cloned into the pGEM-T Easy vector and sequenced. Nucleotide sequences were translated into their putative protein sequences and an unrooted neighbor-joining cladogram was constructed from a ClustalW 2.0 (Larkin *et al.*, 2007) multiple sequence alignment.

WRKY53 protein expression

The Champion pET SUMO protein expression system (Invitrogen, Carlsbad, CA, USA) was used to express the WRKY53 protein (Panavas *et al.*, 2009). Primers TaWRKY53exp_fwd1 and TaWRKY53_exp_rvs1 (Table 3.1) were used to amplify the WRKY53 CDS using a cloned full-length cDNA template previously isolated from GR wheat. The purified amplification product was ligated to the pET Sumo vector and transformed into Mach1-T1^{R} chemically competent *E. coli*. Once the recombinant plasmid pET::W53 was isolated and sequenced to verify the N-terminal in-frame fusion of the WRKY53 CDS with the SUMO tag, the plasmid was transformed into competent BL21(DE3) *E. coli* for expression. Fresh LB medium containing 50 μg mL^{-1} kanamycin and 1% glucose was inoculated at a ratio of 1:50 with overnight culture and grown at 37 °C until mid-log phase (OD_{600} = 0.5). Protein expression was induced with 1mM IPTG and the culture incubated for a further 4.5 h before bacterial cell lysates were prepared. SUMO::TaWRKY53 fusion protein was purified using the N-terminal polyhistidine (6×His) tag and ProBond Ni^{2+}-chelating resin (Invitrogen), following the manufacturer's hybrid purification protocol to ensure maximum solubility and biological activity. Protein yield was determined via a Pierce 660 nm protein assay (Thermo Scientific,

Rockford, IL, USA) and was visualized using 10% polyacrylamide gel electrophoresis. Protein concentration was adjusted to 500 μg mL^{-1} in 30% glycerol.

Electrophoretic mobility shift assay (EMSA)

Potential target promoters for the WRKY53 transcription factor were identified by mining the rice genome for genes with putative functional linkages to LOC_Os05g27730 (*OsWRKY53*) using the RiceNet probabilistic functional gene network (Lee *et al.*, 2011). The Gene Coexpression Analysis tool from the MSU Rice Genome Annotation Project Database (Ouyang *et al.*, 2007) was used to identify genes with expression profiles correlated to that of *WRKY53* during an infection time course with the hemibiotrophic fungal pathogen *Magnaporthe oryzae* (Marcel *et al.*, 2010). Many genes coexpressed with *WRKY53* during pathogen responses are also likely to be coexpressed with *WRKY53* during responses to phloem-feeding insects. The 1 kb upstream promoter regions of all potential candidates were then screened for the presence of W-boxes using the (C/T)TGAC(C/T) consensus motif. Three genes from different functional categories, each with four or more W-boxes, were selected for *in vitro* binding assays with the expressed WRKY53 protein. Synthetic double-stranded DNA probes 80 bp in length were synthesized based on the 1 kb upstream sequence information of these genes (Integrated DNA Technologies, Coralville, IA, USA). A total of 3 μg of purified WRKY53 protein was incubated with 40 ng of each probe in binding buffer (750 mM KCl, 0.5 mM dithiotreitol, 0.5 mM EDTA, 50 mM Tris, pH 7.4) and incubated for 30 min at room temperature. Bovine serum albumin and an aliquot of WRKY53 protein (denatured by boiling for 3 min) were used as non-specific protein controls, whereas a probe based on the 1 kb upstream promoter region of a gene that contained no W-boxes served as a non-specific DNA control. DNA-protein complexes were separated on 6% acrylamide/0.5× TBE non-denaturing gels. Gels were visualized with an Electrophoretic Mobility Shift

Assay Kit (Molecular Probes, Eugene, OR, USA). Nucleic acids were detected with SYBR Green EMSA stain and proteins with SYPRO Ruby EMSA stain. Both stains were visualized using an AlphaImager HP (Cell Biosciences, Santa Clara, CA, USA) and a 537 nm and 620 nm bandpass filter for nucleic acid and protein detection, respectively.

WRKY53 protein-protein interaction assays

Proteins interacting with WRKY53 were identified in yeast two-hybrid assays using Gateway-based bait and prey vectors expressing the *Saccharomyces cerevisiae* GAL4 binding domain (BD) and activation domain (AD), respectively (Nakayama *et al.*, 2002). The pACTGW-*att*R prey vectors consisted of in-frame N-terminal fusions of AD to a previously constructed rice biotic stress cDNA library. This library was created from *Oryza sativa* ssp. *japonica* cv. 'Nipponbare' inoculated with either of the two bacterial pathogens *Xanthomonas oryzae* pv. *oryzae* and *X. oryzae* pv. *oryzicola* and incubated for varying lengths of time before mRNA isolation (Nino-Liu *et al.*, 2005). The pASGW-*att*R bait vector consisted of an in-frame N-terminal fusion of BD to the wheat *WRKY53* coding sequence. Primers attB1-W53_fwd and attB2-W53_rvs (Table 3.3) were used to amplify a full-length clone of *TaWRKY53* from GR cDNA and attach *att*B sites to either end via PCR. The following cycling parameters were used: initial denaturation at 94 °C for 30 s; 35 amplification cycles consisting of denaturation at 94 °C for 20 s, annealing at 60 °C for 20 s, extension at 64 °C for 1:40; final extension at 64 °C for 10 min. The resulting 1,384 bp *att*B PCR fragment was cloned into the pDONR 221 donor vector (Invitrogen) via a BP clonase transposition reaction, forming an entry clone, which was transformed into competent DH5*α* cells and selected for on LB media containing 20 μg mL^{-1} kanamycin. This entry clone was subsequently isolated and recombined with the pASGW-attR destination vector in an LR clonase reaction to form the final pASGW::WRKY53 expression clone, which was transformed into competent DH5*α* cells

and selected for on LB media containing 100 μg mL^{-1} ampicillin. The expression clone was sequenced to verify the integrity of the reading frame. An N-terminal truncated mutant of *TaWRKY53* lacking the first 180 amino acids was amplified using primers attB1-tW53_fwd and attB2-W53_rvs (Table 3.3) and the 884 bp *att*B PCR product was used to construct the pASGW::tW53 bait vector in a procedure analogous to that employed for pASGW::WRKY53. Both the full-length pASGW::WRKY53 and truncated pASGW::tW53 bait vectors were tested for auto-activation and cytotoxicity by transforming into Y2HGold yeast cells using the Frozen-EZ Yeast Transformation II kit (Zymo Research, Orange, CA, USA), and plating onto SD/–Trp media supplemented with either 20 ng mL^{-1} X-*α*-gal (Gold Biotechnology, St. Louis, MO, USA) or X-*α*-gal and 125 ng mL^{-1} Aureobasidin A (Clontech). After co-transformation of 1 μg each of bait and prey vector into Y2HGold yeast cells, the cells were grown on SD/–Leu/–Trp media supplemented with X-*α*-gal. Blue colonies were selected and replica plated onto SD/–Ade/–His/–Leu/–Trp media supplemented with X-*α*-gal and Aureobasidin A (AurA). This selects for the presence of BD vector (–Trp), AD vector (–Leu) and the activation of the four reporter genes *HIS3* (–His), *ADE2* (–Ade), *MEL1* (X-*α*-gal) and *AUR1-C* (AurA). AD vector plasmids were rescued from yeast clones showing positive interactions by scraping colonies from plates into 67 mM KH_2PO_4 and digesting with 30 U of zymolase (Seikagaku, Tokyo, Japan) for 1 h at 37 °C. Digestion was followed by column purification using a QIAprep Spin Miniprep kit (Qiagen, Hilden, Germany). Isolated plasmids were subcloned into DH5*α* *E. coli* in order to obtain a higher yield and tested for the presence of cDNA inserts by PCR with AD_fwd and AD_rvs primers (Table 3.3) before being sequenced.

WRKY53 promoter DNA-protein interaction assays

The 1.2 kb promoter region of WRKY53 was amplified in three segments, using PCR primers attB4-Pw53–400_fwd and attB1R-Pw53–400_rvs, attB4-Pw53–800_fwd and attB1R-Pw53–800_rvs, and attB4-Pw53–1200_fwd and attB1R-Pw53–1200_rvs (Table 3.3) to generate –400:P_{W53}, –800:P_{W53} and –1200:P_{W53} with added Gateway *attB* transposition sites. The following cycling parameters were used: initial denaturation at 94 °C for 2 min; 40 amplification cycles consisting of denaturation at 94 °C for 30 s, annealing at 63 °C for 30 s and extension at 65 °C for 50 s; final extension at 65 °C for 10 min. The *attB* PCR products were gel purified, recombined with the pDONR P4-P1R vector (Invitrogen) in a BP clonase reaction, transformed into chemically induced competent DH5α *E. coli*, and selected for on LB media containing 20 μg mL^{-1} kanamycin. Recombinant entry clones were isolated and recombined with the pDEST-HIS3 and pDEST-LACZ destination vectors (Deplancke *et al.*, 2004) in separate LR clonase reactions to form six expression clones, which were selected for on LB media containing 100 μg mL^{-1} ampicillin. All expression clones were sequenced to verify insert identity. YM4271 yeast cells were sequentially transformed with the corresponding pDEST-HIS3 and pDEST-LACZ expression clones to generate three distinct DNA bait strains placing the *HIS3* and *lacZ* reporter genes under the control of the –400:P_{W53}, –800:P_{W53} or –1200:P_{W53} promoter segments. pDEST-HIS3 and pDEST-LACZ expression clones were respectively linearized with *Xho*I and *Nco*I restriction endonucleases prior to transformation to assist chromosomal integration of the bait constructs at the YM4271 *his3-200* (pDEST-HIS3) and *ura3-52* (pDEST-LACZ) loci. Double recombinant clones were selected on SD/–His/–Ura media. To test autoactivation of the *HIS3* reporter gene and cytotoxicity of the clones, 20 yeast colonies from each double bait strain were replica plated onto SD/–His/–Ura media supplemented with 0, 25, 50, 75 or 100 mM 3-amino–1,2,4-triazole (3-AT) and colony

growth was monitored. To test autoactivation of the *lacZ* reporter gene, yeast colonies were replica plated onto YPDA medium, lifted using Amersham Hybond-NX nylon membrane (GE Life Sciences, Little Chalfont, Buckinghamshire, UK) and snap frozen in liquid nitrogen. The thawed membrane was subsequently soaked in Z buffer (60 mM Na_2HPO_4, 60 mM NAH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$, pH 7.0), 0.18% β-mercaptoethanol and 5% X-gal and incubated overnight. The development of blue colonies indicative of β-galactosidase activity was monitored. Colonies that exhibited minimal growth at the lowest possible 3-AT concentration coupled with minimal β-galactosidase activity were selected as suitable DNA bait strains for use in yeast one-hybrid assays. The $-400{:}P_{W53}$, $-800{:}P_{W53}$ and $-1200{:}P_{W53}$ DNA bait strains were independently transformed with the same biotic stress induced pACTGW-*att*R prey vector library used for yeast two-hybrid assays and selected for on SD/−His/−Leu/−Ura media supplemented with 60 mM 3-AT. AD vector plasmids were rescued from yeast clones showing positive interactions, subcloned into DH5*α E. coli* to obtain a higher yield and tested for the presence of cDNA inserts by PCR with AD_fwd and AD_rvs primers (Table 3.3) before being sequenced.

RESULTS

TaWRKY53 promoter characterization

To obtain upstream promoter information, PCR-based genome walking was performed using nested primers specific for the 5' region of *TaWRKY53*. Of the four restriction endonuclease digest libraries constructed, only amplification from the *Eco*RV, *Pvu*II and *Stu*I libraries resulted in distinct bands after secondary amplification (Figure 3.1); the largest band from the *Pvu*II library provided 1,211 nucleotides of sequence information.

This fragment did not assemble into contigs with any other genome walking fragments, and was the only fragment to exhibit sequence overlap with the 5' region of *TaWRKY53*. This suggests that the large *Pvu*II library fragment represents 1.2 kb of upstream promoter sequence information for *TaWRKY53*. Scrutinizing this sequence for the presence of *cis*-acting regulatory motifs resulted in the discovery of three W-box elements that conform to the (C/T)TGAC(C/T) consensus motif, at –869 –1,064 and –1178 bp upstream of the ATG translation initiation codon (Figure 3.2a). Also present are two ABRE abscisic acid-responsive elements that conform to the (A/C)ACG(C/T)GC motif consensus, at –655 and –875 bp, and a GCC-box ethylene-responsive element that conforms to the AGCCGCC motif at –567 bp upstream. Abscisic acid-responsive genes are upregulated during the greenbug (*Schizaphis graminum*) response of sorghum (Park *et al.*, 2006), whereas GCC-box elements are a hallmark of the promoters of aphid- and pathogen-responsive genes (Rushton and Somssich, 1998; Smith and Boyko, 2007).

TaWRKY53 gene model

Primers were designed based on sequence information from the large *Pvu*II digest library amplicon and the 3' end of accessions EF368357 and EF368364 and used to amplify the entire coding sequence from cDNA (Figure S1) and genomic DNA (Figure S2). A simple sequence alignment (data not shown) with EST accessions EF368364 and EF368357 indicated that the sequence described here is more similar to EF368364. Comparing genomic and cDNA sequences allowed for the mapping of intron-exon boundaries and the construction of a true gene model for *WRKY53* in wheat (Figure 3.2b). The coding region spans five exons, similar to the intron-exon pattern predicted for LOC_Os05g27730, encoding the rice *OsWRKY53*. Exons 3 and 4 encode the two conserved WRKY domains. Exon 3 also includes the zinc finger motif, which conforms to the $CX_{[4-5]}$ $CX_{[22-23]}$HXH consensus sequence for Cys_2–His_2-type zinc fingers. The last

three codons of exon 3 and the first codon of exon 4 encode the nuclear localization signal with consensus sequence KRRK, and similar to *AtWRKY33*, there are several putative phosphorylation sites in the N-terminal region (Figure S3).

WRKY53 allelic diversity

Peptide sequences were predicted from the *TaWRKY53* DNA sequences of nine wheat accessions differing in resistance to *D. noxia* and geographic center of origin. The nine allelic variants assayed were highly similar, with 420 out of 440 amino acid residues being identical across all accessions for a total of 98.8% pair-wise identity (Figure S4). Accession PI361836 had a three-nucleotide deletion relative to the other sequences, resulting in a codon deletion and the removal of a serine residue at the N-terminal of the protein. Half of all amino acid substitutions were between amino acids with similar properties, demonstrating the highly conserved nature of this gene within the sampled pool of genotypes. Most of the non-conservative substitutions were between amino acids with polar and non-polar side groups. An unrooted neighbor-joining cladogram constructed from the multiple sequence alignment indicated that the susceptible US lines 'Yuma' and 'Halt' were the most divergent from the rest of the accessions, including 'Gamtoos-R', which clustered with the Asian landraces (Figure 3.3). PI361836, the only Asian landrace in the sample to exhibit a susceptible phenotype, clustered away from the other Asian landraces. Bootstrap values of 95.7% and 62.9% indicated some support for the separation of these WRKY53 peptide sequences into clades corresponding to *D. noxia* resistance and not geographic center of origin. The highest concentration of amino acid changes separating the two clades occurred at the N-terminal between residues 140 and 160, suggesting that the transactivation domain at the N-terminal of WRKY53 is more prone to changes that might affect its function, whereas the DNA-binding domain remains relatively conserved.

WRKY53 protein expression

Wheat WRKY53 encompasses 439 amino acids and is calculated to be a 47.39 kDa protein. Therefore, the SUMO::TaWRKY53 fusion protein is expected to be ~60 kDa in size. Since attempts to purify recombinant TaWRKY53 using a protocol to maintain its native conformation failed to recover any protein, a denaturing/renaturing hybrid protocol was followed that would allow for the refolding of the protein after binding of the 6×His tag to the Ni^{2+}-chelating resin. Recombinant TaWRKY53 was collected in two major elution fractions (fractions 2 and 3, Figure 3.4), with a total calculated yield in excess of 800 µg.

Electrophoretic mobility shift assays

Potential target promoters for WRKY53 were identified following the rationale that genes that function together have similar expression profiles. RiceNet returned 36 loci linked to LOC_Os05g27730 which encodes *OsWRKY53* (Figure 3.5a), with coherence scores ranging from 1.11 (LOC_Os03g01740) to 3.74 (LOC_Os04g34140). The MSU Gene Coexpression Analysis tool indicated that a total of 62 loci out of 1,161 in the *M. oryzae*-induced dataset were correlated with *OsWRKY53* expression at a very stringent cut-off of between 0.99 and 1 (Figure 3.5b). Defense-related genes upregulated in *OsWRKY53*-overexpressing transgenic rice cells (Chujo *et al.*, 2007) were also included to generate a combined set of 108 potential targets for WRKY53 (Table S1). From this list of 108 loci, 96 had at least one W-box in the 1 kb upstream promoter region, of which 41 had three or more. From this list *chitinase 2* with 4 W-boxes (LOC_Os11g47600), *ORK10* receptor kinase with 5 W-boxes (LOC_Os01g02300,) and the peroxidase *POC1* with 4 W-boxes (LOC_Os07g48050) were selected as candidates for electrophoretic mobility shift assays with the SUMO::TaWRKY53 fusion protein. An unrelated peroxidase *POX5.1*

with no W-boxes in its 1 kb upstream promoter region (LOC_Os07g48040) served as a negative control. These promoter targets and the short dsDNA fragments synthesized from them are summarized in Table 3.4. Gel shift assays using these short synthetic DNA probes and expressed recombinant WRKY53 protein were attempted numerous times, with little success. Both SYBR Green nucleic acid staining and SYPRO Ruby protein staining indicated the presence of large, non-specific DNA-protein complexes at the top of the gel, preventing the native proteins and any possible DNA-protein complexes from moving into the acrylamide (Figure S5). This was not limited to WRKY53 interactions with W-box containing probes, but was also observed with the unrelated control probe and when bovine serum albumin was used as an unrelated control protein. Neither adjusting the ratio of DNA probe to protein nor the total amount of protein used in the assay ameliorated this complication. Conventional EMSA protocols using differentially labeled DNA probes include non-specific competitor DNA in the binding reaction, *e.g.* salmon sperm DNA or synthetic poly d(I:C), to block the effect of non-specific DNA binding (Gaudreault *et al.*, 2008). However, this is usually only necessary when using a complex mix of proteins, such as a nuclear extract, and not a tag-purified protein such as recombinant WRKY53. No mention of the use of competitor DNA was made in the original study detailing dichromatic fluorescence EMSA (Jing *et al.*, 2003). Since SYBR Green detects the presence of double-stranded DNA in a non-sequence specific manner, the addition of extraneous DNA would likely interfere with the assay. The ability of excess amounts of non-specific DNA to prevent the formation of non-specific WRKY53-DNA aggregates was tested, nevertheless. However, several binding reactions which included competitor DNA (either an unrelated 800 bp PCR product, or genomic wheat DNA, or both) were attempted at various concentrations, without success. The influence of the SUMO tag on the ability of the purified protein to bind to DNA has not yet been investigated.

WRKY53 protein-protein interactions

Attempting to use the full-length TaWRKY53 protein in yeast two-hybrid assays resulted in high levels of autoactivation, with all reporter genes being activated even in the absence of the Gal4 AD. This is not surprising, since TaWRKY53 is a transcription factor bearing its own activation domain; the same problem was encountered in a recent study investigating the binding of the *Arabidopsis* ortholog AtWRKY33 to the ATG18a autophagy protein (Lai *et al.*, 2011). To abrogate autoactivity, tW53, a truncated mutant lacking the first 180 amino acids, was fused to the Gal4 BD. Since pASGW::tW53 exhibited no autoactivation (Figure 3.6), this vector was selected for use in yeast two-hybrid assays with the pACTGW-*att*R prey vector library.

More than 200 individual yeast two-hybrid clones were obtained on minimal media (which selects for the presence of both the bait and prey vectors), supplemented with X-*α*-gal as an initial test for putative interactions. False positives were eliminated by replica plating onto more selective media supplemented with X-*α*-gal and AurA, which selects for the activation of all four reporter genes. Five putative interactors were identified from this first round of replica plating, of which only one clone, #318, maintained strong growth and blue color development upon repeated replica plating (Figure 3.6). The plasmids were rescued and sequenced for all five clones. The four dubious clones had significant homology to components of the photosynthetic machinery, which would not normally be in contact with WRKY53, since they are expressed in separate cellular compartments. The failure of the four dubious clones to maintain growth under stringent conditions reflects their false positive status. The cDNA expressed by #318 had significant homology to LOC_Os03g50130, which encodes a putative microsomal glutathione *S*-transferase 3 (Table 3.5).

WRKY53 promoter DNA-protein interactions

To discover possible transcriptional regulators for *TaWRKY53*, yeast one-hybrid assays were conducted with three discrete segments of its 1.2 kb promoter region. Several clones of the –400:P$_{W53}$, –800:P$_{W53}$ and –1200:P$_{W53}$ DNA bait strains were assayed for autoactivity of both the *HIS3* and *lacZ* reporter genes to minimize reporter autoactivation in the absence of prey proteins. Autoactivation can occur when a DNA bait interacts with an endogenous yeast protein, or when multiple copies of a DNA bait construct are integrated into the yeast genome (Deplancke *et al.*, 2004). Several clones for each DNA bait strain exhibited minimal growth at low concentrations of 3-AT, a competitive inhibitor of histidine synthesis (Figure S6). However, all of the –1200:P$_{W53}$ DNA bait strain clones exhibited autoactivation for the *lacZ* locus (Figure S7) and DNA-protein interactions in this strain were subsequently only evaluated based on their ability to activate expression of the *HIS3* gene. Based on these autoactivation assays, one clone from each DNA bait was selected for use in yeast one-hybrid assays with the pACTGW-*att*R prey vector library: –400:P$_{W53}$ clone 1, –800:P$_{W53}$ clone 4 and –1200:P$_{W53}$ clone 18.

A total of 63, 34, and 100 putative interactors with –400:P$_{W53}$, –800:P$_{W53}$ and –1200:P$_{W53}$ respectively were replica plated onto minimal media supplemented with 3-AT. A single positive interactor with –1200:P$_{W53}$ and a total of four positive interactors with –400:P$_{W53}$ were identified (Figure 3.7). No positive interactors were obtained for the –800:P$_{W53}$ DNA bait in this assay. Sequencing results for the five WRKY53 promoter interactors are summarized in Table 3.5.

DISCUSSION

WRKY53 sequence features are remarkably conserved

Alternative splicing is a common feature of plant genomes and more than a quarter of all rice loci encode splicing variants, (Kikuchi *et al.*, 2003) including several *OsWRKY* genes (Xie *et al.*, 2005). However, LOC_Os05g27730, encoding *OsWRKY53*, is predicted to produce only a single splicing variant, and our primers for amplifying *TaWRKY53* in wheat were unable to detect more than a single transcript. The three W-boxes present in the promoter of TaWRKY53 are similar in number and orientation to those found in its orthologs in other plant species, including the *OsWRKY53* promoter (Chujo *et al.*, 2009), *AtWRKY33* (Lippok *et al.*, 2007) and the parsley (*Petroselinum crispum*) *PcWRKY1* promoter (Turck *et al.*, 2004), although the W-boxes are located much further upstream in *TaWRKY53*. This remarkable level of inter-species regulatory element conservation, coupled with the lack of TaWRKY53 sequence diversity observed among divergent wheat accessions, implies strong regulatory control of this WRKY and a pivotal role in plant stress resistance.

Intact W-boxes are required for elicitor-responsiveness of *OsWRKY53* (Chujo *et al.*, 2009) and pathogen-responsive induction of *AtWRKY33* (Lippok *et al.*, 2007). *TaWRKY53* may be self-regulated or regulated by other WRKY proteins during defense responses. Once induced to very high levels, the parsley ortholog PcWRKY1 binds W-boxes in its own promoter (Turck *et al.*, 2004) and it is possible that TaWRKY53 exhibits the same capacity for negative self-regulation. The presence of a GCC-box ethylene responsive element is consistent with the discovery that *AtWRKY33* expression is significantly reduced in the *ein2* mutant, defective in ET-response signaling (Li *et al.*, 2011).

OsWRKY53 coexpression networks and identifying TaWRKy53 targets

We selected three genes with distinct functions in plant resistance responses as potential targets for regulation by *TaWRKY53*. The rice *ORK10* gene is a homologue of *Lrk10* in wheat, a Ser/Thr protein kinase associated with defense against biotrophic rust fungi in wheat (Feuillet *et al.*, 1997) and other cereals (Cheng *et al.*, 2002). In response to the hemibiotrophic rice blast fungus, *M. oryzae*, *ORK10* is co-induced with *OsWRKY53* and several genes involved in the perception and degradation of chitin (Marcel *et al.*, 2010). We found five W-boxes in its promoter region, which implies that *ORK10* forms an integral component of WRKY transcriptional networks.

Chitinase 2 is highly upregulated in *OsWRKy53* overexpressing lines (Chujo *et al.*, 2007) and its promoter region contains four putative W-boxes. Additionally, chitinases are selectively expressed in the apoplast of resistant wheat during *D. noxia* infestation (Van der Westhuizen *et al.*, 1998), which requires the expression of *TaWRKY53* to be resistant (Van Eck *et al.*, 2010). Although we were unable to demonstrate a direct interaction between TaWRKY53 protein and a chitinase promoter in this study, chitinous compounds secreted by fungi and phloem-feeding insects are major elicitors of plant defense responses (Akimoto-Tomiyama *et al.*, 2003; Ramonell *et al.*, 2005). The amplification of resistance signaling responses could therefore be facilitated by modulation of WRKY transcription factors that bind to the W-boxes present in the promoters of chitinase genes.

The cationic peroxidase *POC1* is highly expressed in the apoplast of mesophylll cells infected with *X. oryzae* pv. *oryzae* (Young *et al.*, 1995). Apoplastic peroxidases and chitinases are induced in wheat plants genetically resistant to *D. noxia* upon aphid feeding (Van der Westhuizen *et al.*, 1998) and also in response to the leaf rust *Puccinia triticina* (Anguelova-Merhar *et al.*, 2002); it is thought that peroxidase activity in the

intercellular space might help cross-link cell wall components, interfering with aphid probing or fungal ingress.

TaWRKY53, GST and regulation of the oxidative burst

Our yeast two-hybrid analysis revealed a microsomal glutathione *S*-transferase 3 as a putative interactor with the WRKY53 protein (Figure 3.6). Glutathione *S*-transferases (GSTs) are a large class of highly abundant glutathione conjugating enzymes from at least four paraphyletic gene families (Pearson, 2005). GSTs are well-known for their role in ameliorating oxidative damage in plants, with well-described functions in herbicide resistance (Cummins *et al.*, 2011) and pathogen responses (Lieberherr *et al.*, 2003). GSTs, along with a whole suite of other enzymes with antioxidant activity, including superoxide dismutases, catalases and peroxidases, are responsible for scavenging free radicals in the wake of the stress-induced oxidative burst (Gill and Tuteja, 2010). These enzymes have a demonstrated role in protecting wheat during the oxidative burst induced by *D. noxia* feeding (Moloi and Van der Westhuizen, 2008). There is evidence that GSTs are an essential part of WRKY transcriptional networks. A GST from potato exhibits a conserved W-box in its promoter (Hahn and Strittmatter, 1994) and coexpression of a GST with a WRKY transcription factor was reported in response to ozone treatment of European beech saplings (Olbrich *et al.*, 2005). Overexpression of a strawberry *WRKY* gene in *Arabidopsis* led to the increased production of H_2O_2 and also massive induction of GST after inoculation with *Pseudomonas syringae* pv. tomato DC3000 (Encinas-Villarejo *et al.*, 2009). SA-responsive upregulation of a GST was reduced in *OsWRKY45* knockdown plants (Shimono *et al.*, 2007). Many GSTs are induced in SA-mediated pathogen responses (Sappl *et al.*, 2004) and three SA-regulated GSTs were induced within 36 hours of green peach aphid (*Myzus persicae*) infestation of *Arabidopsis* (Couldridge *et al.*, 2007). GSTs were also isolated from wheat EST libraries

enriched for *D. noxia* responsive transcripts (Botha *et al.*, 2005), consistent with their involvement in aphid defense.

LOC_03g50130 is annotated as a putative microsomal glutathione *S*-transferase 3, based on significant homology to the human *MGST3* gene. MGSTs are part of the larger MAPEG protein family (membrane-associated proteins in eicosanoid and glutathione metabolism) and are involved in detoxifying foreign compounds and the metabolites of oxidative stress (Jakobsson *et al.*, 1999). These proteins differ considerably from the better-studied cytoplasmic and mitochondrial GSTs, being much smaller at 150–160 amino acids in length, and functioning mostly as trimers (Pearson, 2005). In addition to glutathione *S*-transferase activity, mammalian MGSTs also exhibit glutathione-dependent peroxidase activity, capable of reducing membrane-damaging lipid peroxidation and free H_2O_2 (Gill and Tuteja, 2010; Jakobsson *et al.*, 1999); plant GSTs with glutathione peroxidase activity have been previously described in pea (*Pisum sativum*) (Edwards, 1996). To our knowledge, our study is the first report indicating a possible role for membrane-bound GSTs in defense against phloem-feeding insects. Considering that *D. noxia* feeding induces chlorosis and oxidative damage to cereal leaves (Ni *et al.*, 2001; Ni and Quisenberry, 2003) and that we previously found TaWRKY53 to be essential for aphid resistance, our yeast two-hybrid data provide evidence that membrane-bound glutathione *S*-transferases are able to alter the ROS response in a TaWRKY53-mediated way. This could be achieved either through the induction of detoxifying gene products to protect the photosynthetic machinery from free-radical damage, or by quenching runaway ROS production during the hypersensitive response.

New potential regulators of TaWRKY53 expression

LOC_Os07g36190 encodes an isopentenyl-diphosphate delta-isomerase that interacted with the –1200:P$_{W53}$ promoter region of *TaWRKY53* in yeast one-hybrid assays (Figure 3.7). Isopentenyl-diphosphate delta-isomerase (IDI) catalyzes the conversion of isopentenyl-diphosphate (isopentenyl-pyrophosphate, IPP) to dimethylallyl-diphosphate (dimethylallyl-pyrophosphate, DMAPP) and is a key component of the mevalonate pathway leading to the synthesis of terpenoids, including several phytoalexins (Okada *et al.*, 2008). IDIs are expressed in the cytosol and other subcellular compartments such as plastids and mitochondria, but not the nucleus (Phillips *et al.*, 2008), and no evidence of DNA-binding activity has been reported in the literature. The interaction of a rice IDI with the promoter of *TaWRKY53* in our yeast one-hybrid assay likely represents a false positive, since these two components would not normally interact *in planta*. This demonstrates the importance of supporting evidence from the scientific literature or additional experimental confirmation before drawing conclusions from yeast hybrid assays.

LOC_Os08g42850 encodes an FKBP-type peptidyl-prolyl *cis-trans* isomerase (PPI) that interacts with the –400:P$_{W53}$ promoter region of *TaWRKY53* in yeast one-hybrid assays (Figure 3.7). FK506-binding proteins, or FKBPs, are named after their ability to bind the immunosuppressant drug FK506 in mammals, and have important chaperone functions during protein folding (Fischer *et al.*, 1989). There are 29 FKBP loci in the rice genome; the FKBP encoded by LOC_Os08g42850 has been recently assigned the gene name *OsFKBP16-3* (Ahn *et al.*, 2010; Gollan and Bhave, 2010). Some rice FKBPs are expressed in the nucleus, but *OsFKBP16-3* possesses an N-terminal chloroplast-targeting signal sequence (Ahn *et al.*, 2010) and its expression has previously only been detected in the thylakoid lumen (Gollan and Bhave, 2010). *OsFKBP16-3* has tantalizing functions in osmotic stress tolerance (Ahn *et al.*, 2010), but it is not known

whether it is able to directly interact with the promoters of nuclear genes such as *TaWRKY53*. Interestingly, a PPI was highlighted in a recent study as one of only four genes that are induced by SA-analogue treatment in wild-type *Arabidopsis*, but that fail to accumulate in *wrky33* mutants (Qiu *et al.*, 2008). This supports our yeast one-hybrid data, suggesting that peptidyl-prolyl isomerase activity forms part of the *WRKY53* transcriptional network.

LOC_Os01g72100 encodes OsCML10, a calmodulin-related calcium sensor protein that demonstrated interaction with the $-400{:}P_{W53}$ promoter region of *TaWRKY53* in our yeast one-hybrid assays (Figure 3.7). Ca^{2+}-binding is a feature of a diverse set of gene families with diverse signaling functions, including calmodulins (*CaM*), CaM-like *CML* genes, *CPK* Ca^{2+}-dependent protein kinases and *CBL* calcineurin B-like genes (Reddy and Reddy, 2004). The *CML* genes are closely related to *CaM* genes, consisting almost entirely of Ca^{2+}-binding EF-hand domains (Boonburapong and Buaboocha, 2007; Popescu *et al.*, 2007). The *Arabidopsis* genome has six *CaM* genes and 50 *CML* genes (McCormack and Braam, 2003), whereas the rice genome has five *CaM* genes and only 32 *CML* genes (Boonburapong and Buaboocha, 2007). *OsCML10* is expressed in leaves, roots and panicles, and shares 46% identity with *OsCaM1* (Boonburapong and Buaboocha, 2007) but its responsiveness to biotic and abiotic stress has not been ascertained. Although direct DNA-binding by a CML protein has not been demonstrated, they do interact with transcription factors (Popescu *et al.*, 2007; Reddy and Reddy, 2004). Examples of these are the *CAMTA* CaM-binding transcription activators in *Arabidopsis*. Several genes involved in pathogen defense are upregulated in *camta3* mutants, including *AtWRKY33* (Galon *et al.*, 2008), suggesting that CAMTAs suppress the biotic stress response. Yeast possesses an array of different CaM-binding proteins (Dos Santos *et al.*, 1997; Liu *et al.*, 1991). It is therefore possible that *OsCML10* interacts with endogenous yeast transcription factors to activate reporter gene

transcription via the *TaWRKY53* promoter in our yeast one-hybrid assays. Whether this has any relevance to *in vivo* interaction between *TaWRKY53* and wheat calmodulin-like calcium sensor proteins, remains to be determined.

LOC_Os07g47640 encodes an ultraviolet B-repressible protein that interacts with the –400:P_{W53} promoter region of *TaWRKY53* in yeast one-hybrid assays (Figure 3.7). There is mounting evidence for UV responsiveness in *WRKY* genes (Jang *et al.*, 2010): *AtWRKY33* mRNA rapidly accumulates 24 hours after exposure of *Arabidopsis* seedlings to UV-containing white light (Lippok *et al.*, 2007); UV-B radiation increases *NtWRKY2* transcript abundance in *Nicotiana longiflora* (Izaguirre *et al.*, 2003); and *OsWRKY89* is not only strongly induced within 12 hours of UV-B exposure, but overexpression of *OsWRKY89* affords increased tolerance to UV radiation (Wang *et al.*, 2007). Interestingly, UV-responsive *WRKY* genes are also implicated in defense against fungal pathogens, phloem-feeding insects and chewing insects (Izaguirre *et al.*, 2003; Wang *et al.*, 2007). In fact, there is significant overlap in plant response strategies to ultraviolet radiation and biotic stressors, including MAP kinase cascades, JA/ET signaling, and the generation of ROS (Stratmann, 2003). In a study on the effect of ozone (another oxidative abiotic stressor) on saplings of European beech (*Fagus sylvatica*), an ultraviolet-B repressible protein was downregulated, whereas the expression of a *WRKY*-like transcription factor was significantly induced (Olbrich *et al.*, 2005). Our yeast one-hybrid interaction assays indicate that ultraviolet-B repressible proteins are able to bind to the promoters of *WRKY* genes. In the case of *TaWRKy53*, this represents another layer of stress-responsive transcriptional regulation, with ultraviolet-B repressible proteins acting as potential *trans*-acting negative regulators.

Do aphids mobilize nutrients by stimulating senescence?

LOC_Os04g45834 encodes a DUF584 domain-containing protein that strongly activates the –400:P_{W53} promoter region of *TaWRKY53* in yeast one-hybrid assays (Figure 3.7).

The DUF584 domain is one of more than 3,000 Domain of Unknown Function families collected in the Pfam database; currently there are 201 such DUF584 domain-containing proteins listed there. Although very little research has been conducted on DUF584 proteins to date, work on HvS40, a DUF584 protein in barley, suggests a key role in senescence. Senescence is an ordered, genetically controlled process in which the photosynthetic apparatus is systematically dismantled and free amino acids are moved to sink tissues such as newer leaves or developing seeds (Gregersen *et al.*, 2008). Aphids could possibly exploit senescence responses by intercepting remobilized amino acids transported in the phloem. The barley HvS40 protein contains a DUF584 domain and is highly induced in the mesophyll cells of senescing leaves (Krupinska *et al.*, 2002). The barley HvS40 is induced by JA, and both HvS40 and the *Arabidopsis* ortholog AtS40-3 are induced by SA and attack by various fungal pathogens; both of these proteins have exhibited DNA-binding capacity (Fischer-Kilbienski *et al.*, 2010; Krupinska *et al.*, 2002). AtS40-3 regulates the expression of a WRKY transcription factor, *AtWRKy53*, which has been shown to be specifically associated with early events in senescence (Fischer-Kilbienski *et al.*, 2010). It is important to note that the Arabidopsis *AtWRKY53* is not orthologous to *OsWRKY53* and *TaWRKY53* in rice and wheat (which are instead orthologous to *AtWRKY33*). However, *TaWRKY53* itself has been shown to be upregulated in senescing leaves of wheat (Wu *et al.*, 2008).

The activation of AtWRKY53 by AtS40-3 differs between early and late stages of senescence (Fischer-Kilbienski *et al.*, 2010), which suggests strong regulatory control of the exact sequence of events. During late stage senescence, amino acids from catabolized chloroplast proteins are recycled into double membrane autophagosomes in the cytoplasm by ATG autophagy proteins (Gregersen *et al.*, 2008). Interestingly, the autophagy protein ATG18a interacts with *AtWRKY33* in the nucleus and autophagy mutants develop senescence-like chlorotic symptoms and increased protein degradation

upon infection with *B. cinerea* (Lai *et al.*, 2011). Catabolic enzyme assays have shown that chlorosis induced by *D. noxia* infestation of wheat differs from natural senescence (Heng-Moss *et al.*, 2003; Ni *et al.*, 2001). For aphids, as vascular feeders, to obtain access to chloroplast proteins, they require the capacity to induce a highly modulated form of senescence. This aphid-induced senescence would maintain the nutrient mobilization events of chloroplast breakdown and chlorosis that occur during early stage senescence, and delay late stage cellular collapse for as long as metabolically possible. It is well-documented that aphids can change the nutritional composition of phloem sap to have higher levels of free amino acids (Sandström *et al.*, 2000). The ability to create a state of chlorosis has major impacts on aphid fitness, since mutant clones of *S. graminum* unable to induce chlorosis in wheat perform better on plants previously infested by *S. graminum* genotypes that do induce chlorotic lesions (Dorschner *et al.*, 1987). The enzyme activities detected in *D. noxia* saliva induce more oxidative stress in cereals than the saliva of *R. padi*, a species that does not induce chlorosis (Ni and Quisenberry, 2003). Our yeast one-hybrid data suggest that S40-like proteins from cereals can also regulate the expression of *TaWRKY53*, similar to how AtS40 regulates the expression of the senescence-related *AtWRKY53* in *Arabidopsis*. Since we have shown that *TaWRKY53* is upregulated in resistant wheat leaves that do not exhibit aphid-induced chlorosis, we speculate that a DUF584 protein might act as a negative regulator of *TaWRKY53* during compatible interactions with *D. noxia*.

TaWRKY53 in aphid defense

We have presented here several lines of evidence that lead us to hypothesize a role for *TaWRKY53* in modulating the oxidative burst and preventing chlorosis during the *D. noxia* resistance response. Aphid-derived chitinous oligosaccharides trigger a basal resistance response, similar to the MTI (MAMP-triggered immunity) elicited by fungal-

derived chitinous compounds. However, plant perception of aphid salivary proteins in a gene-for-gene mechanism involving the wheat *Dn* resistance genes (Lapitan *et al.*, 2007) would lead to a more effective and coordinated defense response. This is similar to the ETI (effector-triggered immunity) induced by microbial pathogens, and involves ROS, localized hypersensitive cell death, and changes to the cell wall architecture (Botha *et al.*, 2005).

Since many chitinase genes have W-boxes in their promoters and *OsWRKY53* is chitin inducible, and *TaWRKY53* is also required for *Dn*-mediated aphid defense (Van Eck *et al.*, 2010), we conclude that *TaWRKY53* is potentially involved in both basal and *Dn*-mediated aphid resistance responses. It has been suggested that WRKY transcription factors may facilitate cross-talk between multiple signaling pathways (Smith and Boyko, 2007). Based on genes that are coexpressed with *OsWRKY53* during biotic stress that also harbor W-box elements, *TaWRKy53* is an important upstream regulator of ROS and the hypersensitive response.

TaWRKY53 is largely activated by MAPK signaling cascades, and its activity during the progression of the defense response is enhanced or attenuated by various components of the stress response repertoire. We have demonstrated that this regulation can occur at a transcriptional level by the binding of various proteins involved in senescence, Ca^{2+} signaling and SA responsiveness to the *TaWRKY53* promoter; and also at a post-translational level by protein-protein interactions with the TaWRKY53 transcription factor. We identified glutathione *S*-transferase as such a modulator, which we expect to act in protecting the plant from the harmful effects of its own oxidative burst by negatively regulating the activity of TaWRKY53.

Table 3.1. Primers for the characterization of *WRKY53* in wheat

Purpose	Primer ID	Primer Sequence
Genome walking	TaWRKY53_GSP1	CGCCAGACCCTGATAGAAGCTCAGTCAAGG
	TaWRKY53_GSP2	AAGGAGGACATGGCGATCGACGCGACGGAA
Full-length clones	TaWRKY53_CDS_fwd	CCCTGCTCCTCCCGTCGCTC
	TaWRKY53_CDS_rvs	CGTGGACCCACATGTAAACGCCA
Protein expression	TaWRKY53exp_fwd	ATGTCCTCCTCCACGGGGAGCTTGGACC
	TaWRKY53exp_rvs	GCCGCGGCCTAGCCTGCCTAGCTAGCAG

Table 3.2. Wheat lines used for *TaWRKY53* allelic diversity assay

Accession	Source country	*D. noxia* susceptibility
Gamtoos-R	South Africa	resistant
Yuma	USA	susceptible
Halt	USA	susceptible
PI221482	Afghanistan	resistant
PI361836	Afghanistan	susceptible
PI621394	Iran	resistant
PI622825	Iran	resistant
PI623081	Iran	resistant
PI623836	Iran	resistant

Table 3.3. Primers used for the construction of yeast-hybrid vectors

Primer ID	Primer Sequence
attB1-W53_fwd	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCATGTCCTCCTCCACGGGGAGCTTG
attB2-W53_rvs	GGGGACCACTTTGTACAAGAAAGCTGGGTCCTACTAGCAGAGGAGCGACTCGACGAA
attB1-tW53_fwd	GGGGACAAGTTTGTACAAAAAAGCAGGCTTATACAATTGGAGGAAGTACGGGCAG
attB4-Pw53–400_fwd	GGGGACAACTTTGTATAGAAAAGTTGTCTCGATTGATTGCCCGCACCAAA
attB1R-Pw53–400_rvs	GGGGACTGCTTTTTTGTACAAACTTGACCGACGGTACATGCCATAGGTCC
attB4-Pw53–800_fwd	GGGGACAACTTTGTATAGAAAAGTTGCGTGTTGGTGCAGCCATCTCGTAT
attB1R-ppw53–800_rvs	GGGGACTGCTTTTTTGTACAAACTTGTGCGGGGTTTGTTTTACTCTGGAA
attB4-Pw53–1200_fwd	GGGGACAACTTTGTATAGAAAAGTTGATCAGGGTCTGGCGTAGTCAGGTG
attB1R-Pw53–1200_rvs	GGGGACTGCTTTTTTGTACAAACTTGGCATGGTACATCCCCGACCTGAGA
AD_fwd	CTATTCGATGATGAAGATACC
AD_rvs	GTGAACTTGCGGGGTTTTTCA

Table 3.4. Electrophoretic mobility shift assay probes

Locus ID	Annotation	EMSA fragment[a]
LOC_Os11g47600	*chitinase 2*	AGCCTCACGTTTCGTCCTGATTGCAAGTTG**TTGACT**TAAAT **TTGACT**TGTCTCGGAACAAAACAATAACCTGCAGTCCGT
LOC_Os01g02300	*ORK10* kinase	ATCT**GGTCAA**CAATGTATTACACACTGCT**TTGACT**ACTTCC CCCAAAAAAGTACACACTGCT**TTGACT**CA**GGTCAA**ACTT
LOC_Os07g48050	*POC1* peroxidase	ACGTAAATTTTTTGAATAAGACAAAT**GGTCAA**ACATGTAAG AAAAGAA**AGTCAA**CGGCGTCATCTATTTAAAAAACGGAT
LOC_Os07g48040	*POX5.1* peroxidase	TTCCATTATATGATGATTAAATTGGGAAAGAGAGAGTATTA GCTTTTTCAAATAAATATAGGGGGGGGTGGTTCAGCAAT

[a]The presence of W-boxes is indicated in bold.

Table 3.5. Yeast hybrid interactors

Interaction	Bait	Clone ID	Homology[a]	E-value
One-hybrid	–400:P_{W53}	9	LOC_Os01g72100 *OsCML10* calmodulin-related calcium sensor protein	$3.9e^{-59}$
		31	LOC_Os08g42850 FKBP-type peptidyl-prolyl *cis-trans* isomerase	$3.9e^{-104}$
		107	LOC_Os07g47640 ultraviolet B-repressible protein	$1.3e^{-132}$
		111	LOC_Os04g45834 DUF584 domain-containing protein	$1.1e^{-10}$
	–1200:P_{W53}	6	LOC_Os07g36190 *ipp2* isopentenyl-diphosphate delta-isomerase	$1.0e^{-105}$
Two-hybrid	tW53	318	LOC_Os03g50130 microsomal glutathione *S*-transferase 3	$3.9e^{-59}$

[a]Homology based on BLASTn searches of the MSU Rice Genome Annotation Project database.

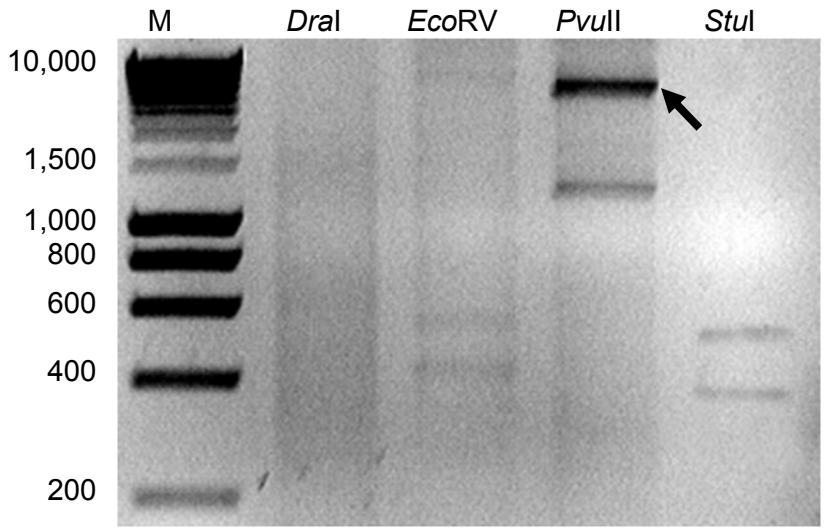

Figure 3.1. Bands from genome walking. The large, distinct band from the *Pvu*II library (indicated by an arrow) spans the *TaWRKY53* upstream promoter area.

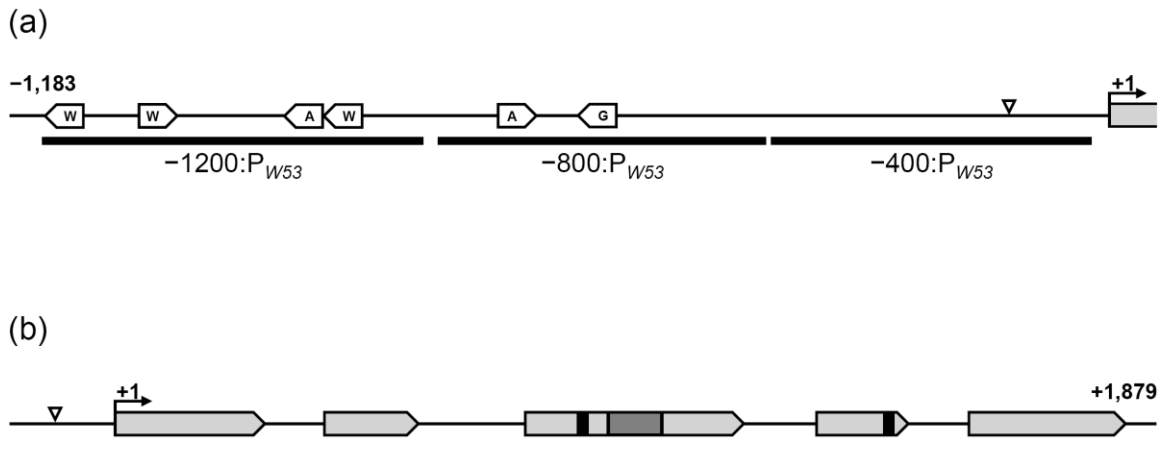

Figure 3.2. A gene model for *TaWRKY53*. Exons are represented by grey arrows. The putative transcription start site is indicated by an open arrow. (a) The 1.2 kb promoter region. Putative *cis*-acting regulatory elements indicated by open arrows: A, ABRE abscisic acid-responsive element; G, GCC-box ethylene-responsive element; W, W-box WRKY transcription factor binding motif. The regions amplified for use in yeast one-hybrid assays are indicated by horizontal black bars. (b) Intron-exon map. The *TaWRKY53* gene spans five exons. The two conserved WRKY domains are indicated by black bars; the zinc finger motif is indicated by a dark grey bar.

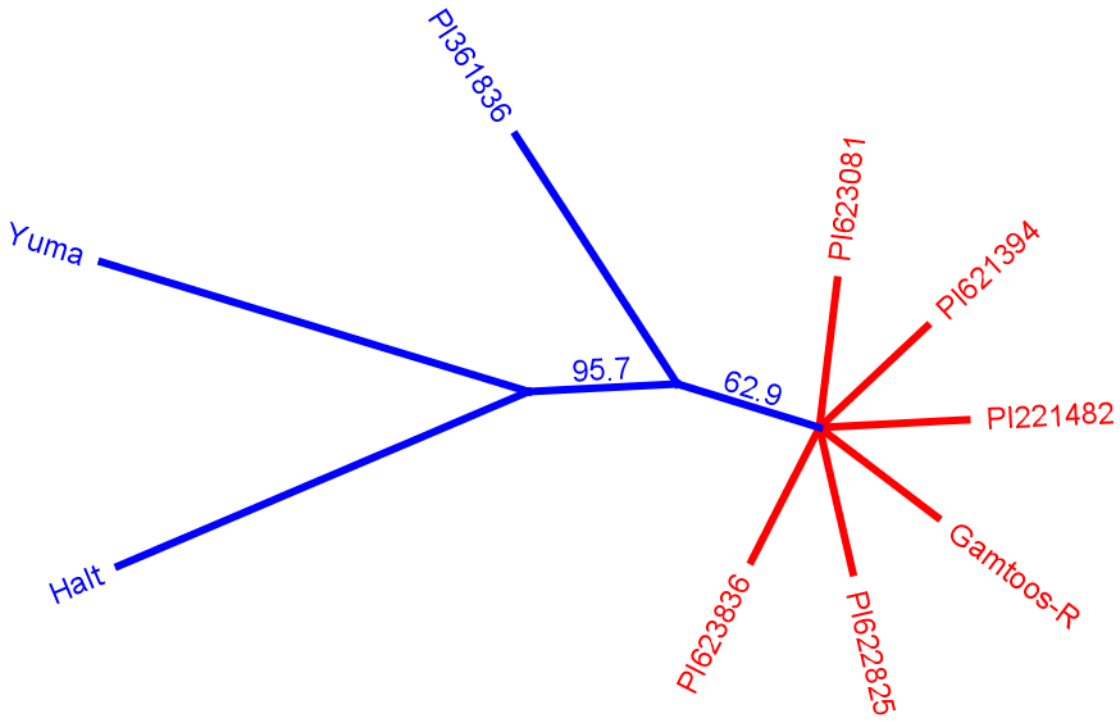

Figure 3.3. Unrooted neighbor-joining consensus cladogram constructed from a multiple sequence alignment of TaWRKY53 protein sequences from 9 different accessions. Stability of groups is indicated by bootstrap values. These suggest some support for the separation of resistant (red) and susceptible (blue) accessions into distinct clades.

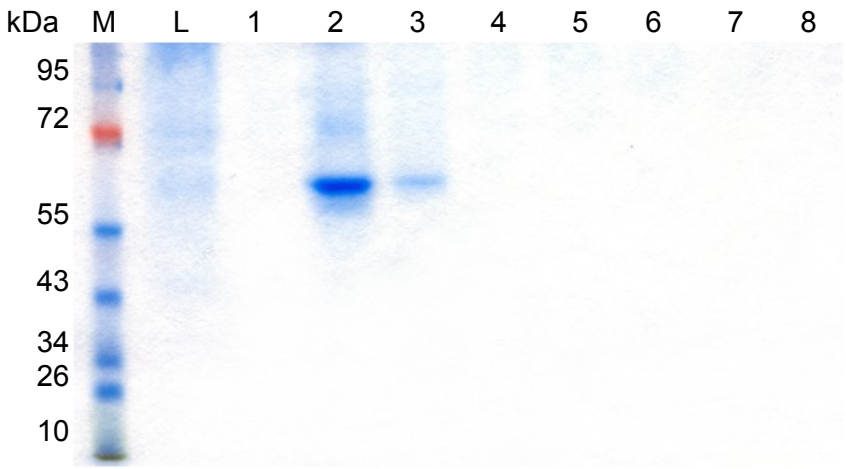

Figure 3.4. Recombinant WRKY53 protein expression. M, size marker; L, crude bacterial lysate; 1–8, sequential elution fractions, with the 60 kDa recombinant WRKY53 protein being captured in the second and third elutions.

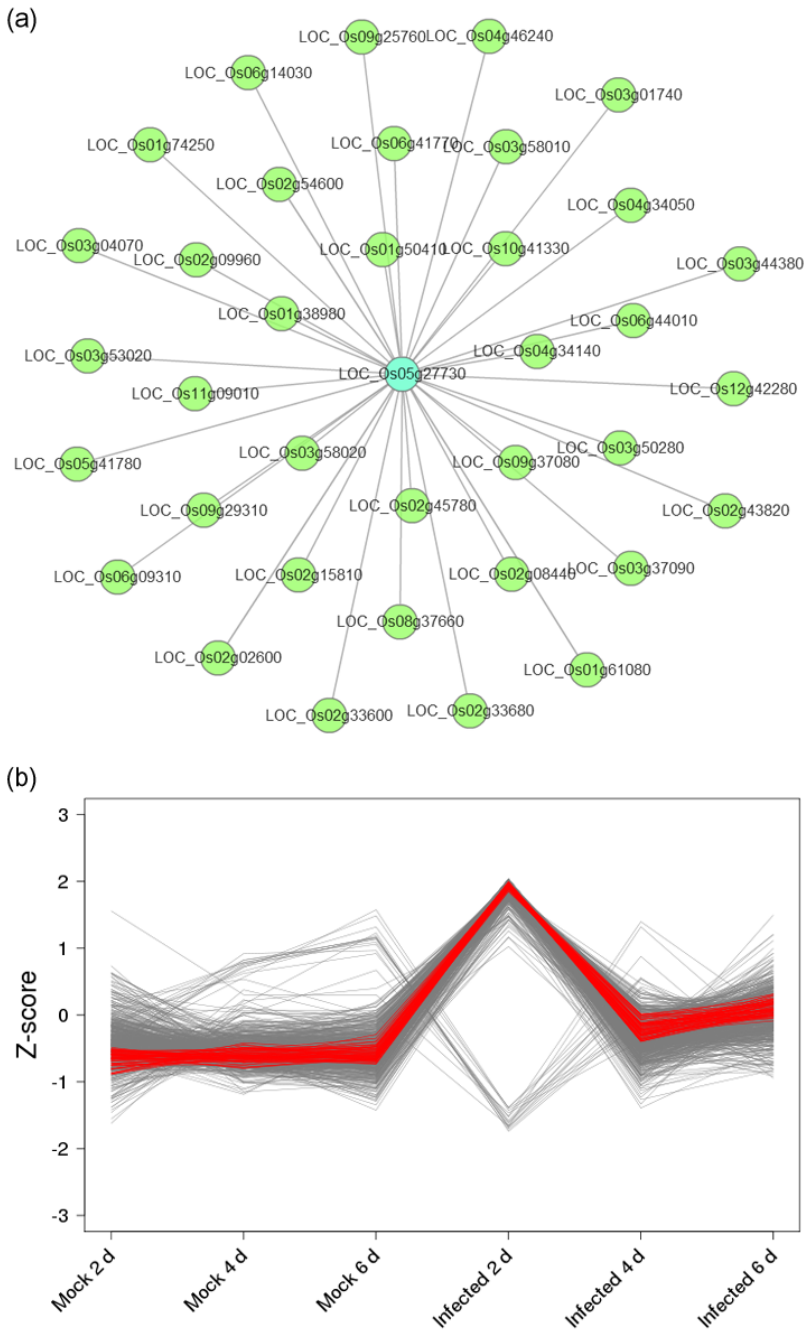

Figure 3.5. Bioinformatically determined *WRKY53* coexpression networks. (a) RiceNet CytoScape probabilistic functional gene network indicating the presence of 36 loci coexpressed with LOC_Os05g27730, encoding OsWRKy53. (b) MSU Rice Genome Annotation Project Gene Coexpression Analysis. The 62 out of 1,161 loci in the *M. oryzae* infection-induced dataset that were coregulated with LOC_Os05g27730 at a correlation > 0.99 are indicated in red.

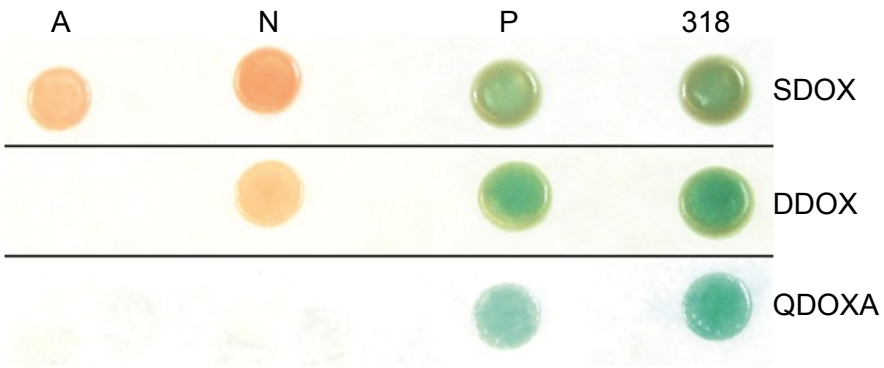

Figure 3.6. Yeast two-hybrid interactions. All colonies express the truncated WRKY53 mutant from the pASGW::tW53 vector. Blue color indicates the activation of the *MEL1* gene. A, autoactivation control; N, negative control; P, positive control; 318, positive interactor, a microsomal glutathione-*S*-transferase. SDOX, single dropout SD/–Trp/X-*α*-gal media; DDOX, double dropout SD/–Leu/–Trp/X-*α*-gal media; QDOXA, quadruple dropout SD/–Leu/–Trp/–Ade/–His/X-*α*-gal/AurA media.

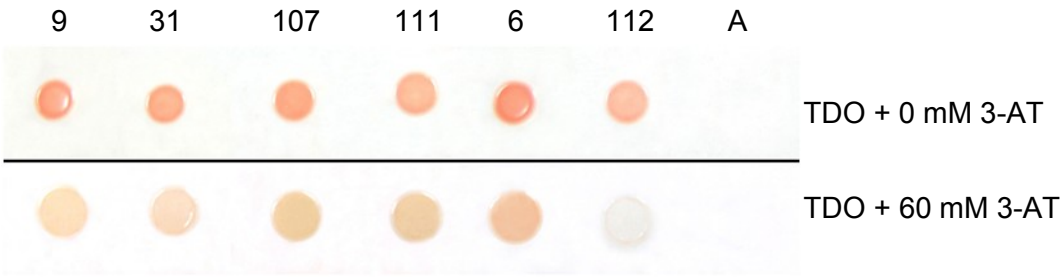

Figure 3.7. Yeast one-hybrid interactions. Colonies 9–111 have the HIS3 and lacZ reporter genes under the control of the –400:P_{W53} promoter segment. Colonies 6 and 112 have the reporter genes under the control of the –1200:P_{W53} promoter segment. Colony 112 harbors an empty prey protein vector, and acts as a negative control. The identities of the interactors are summarized in Table 3.5. A, autoactivation control; TDO, triple dropout SD/–His/–Leu/–Ura media; 3-AT, 3-amino–1,2,4-triazole.

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CHAPTER 4:

THE SECRETED SALIVARY PROTEOME OF TWO RUSSIAN WHEAT APHID BIOTYPES

SUMMARY

Since aphids use their saliva to modulate plant defense responses, we compared the salivary proteins secreted by two Russian wheat aphid biotypes. Saliva was collected from biotypes RWA1 and RWA2 using feeding cages and an artificial sucrose diet and compared using fluorescence differential gel electrophoresis (DIGE). Differentially expressed proteins were excised from 2D gels and subjected to mass spectrometry. Proteins were assigned identities via homology to gene models in the *Acyrthosiphon pisum* genome sequence and a database of *Schizaphis graminum* short sequence reads. Our data uncovered several proteins previously reported from aphid saliva, including glucose dehydrogenase, aminopeptidase and apolipophorin. However, most proteins were of bacterial origin, inoculated into the artificial diet by the aphids themselves. Subsequent iTRAQ and MuDPIT analysis suggested that the contribution of aphid-derived proteins to the total protein pool in our samples was small, but that the number of aphid-specific proteins identified corresponds to previous reports for salivary proteins collected from artificial diets. We hypothesize that most aphid species share a few common proteins in their secreted saliva, but are able to modulate the exact composition. Based on the discrepancy between secreted proteins and proteins with

secretion signals isolated from salivary glands, we speculate that some proteins may be specific to E2 salivation, which was not collected with our experimental setup. Russian wheat aphids likely inoculate plants with microbes or microbial peptides, thereby eliciting inappropriate responses that accelerate disease symptoms such as chlorosis to mobilize nitrogen into the phloem.

INTRODUCTION

Aphid feeding behavior

Aphids feed from single phloem sieve elements for an extended period of time, during which they keep these cells alive and modulate plant defense responses. Factors present in aphid saliva interact with the plant to establish successful feeding. Aphids produce two distinct kinds of saliva (Miles, 1959): a gel-like saliva that hardens to form the stylet sheath, and a soluble watery saliva that is injected into the plant to help suppress plant defenses (Will *et al.*, 2007). Gel-like saliva is secreted onto the leaf surface and the stylets are inserted through this droplet into the epidermis, intermittently secreting more gel-like saliva to form the salivary sheath around the stylets as they probe intercellularly towards the vascular bundle. The salivary sheath is composed of a combination of proteins, phospholipids and conjugated carbohydrates (Giordanengo *et al.*, 2010; Smith and Boyko, 2007). Aphids secrete some watery saliva during probing as the stylet tips puncture cells on their way to the final feeding site, but the majority of watery saliva is secreted into the sieve elements during phloem feeding. Electronic penetration graph (EPG) analysis has demonstrated that two main events are accompanied by the secretion of watery saliva into the final sieve element. The first is E1 salivation, a continual injection of saliva into the sieve element; E1 salivation is thought to be the event during which aphid-derived effector proteins are injected to modulate the nutritional content of

the phloem and also inhibit plant defense responses (Goggin, 2007). E1 salivation is rapidly followed by E2 salivation, which is mixed with phloem sap and immediately ingested without entering the plant cell; E2 salivation is thought to help prevent proteins from coagulating in the food canal (Tjallingii, 2006). Analysis of salivary gland EST sequences and salivary extracts from different aphids indicates the presence of shared and species-specific proteins (Bos *et al.*, 2010; Cooper *et al.*, 2011). Such differences in saliva composition may reflect differences in host range (Carolan *et al.*, 2009) and virulence.

The composition of aphid saliva

The first investigations into the constituents of aphid saliva relied on aphids probing filter paper soaked in enzyme substrates, and the subsequent separation of hydrolysis products via paper chromatography (Adams and McAllan, 1956). Several salivary enzyme activities have since been described. Oxidoreductases and hydrolases are two major classes of enzyme activity found in aphid saliva. The oxidoreductases include enzymes such as polyphenoloxidase, glucose oxidase and peroxidase, thought to detoxify defensive phytochemicals (Cherqui and Tjallingii, 2000; Ma *et al.*, 2010). Oxidoreductases in the saliva of caterpillars perform salivary-specific post-translational modification of plant proteins, including dephosphorylation of *LOX2*, an important component of JA-mediated responses to insect herbivory (Thivierge *et al.*, 2010). Polygalacturonase, sucrase and pectinase are examples of hydrolases reported in aphid saliva; these hydrolyzing enzymes are thought to suppress wounding responses, aid stylet penetration, and induce beneficial changes in plant metabolism (Goggin, 2007; Harmel *et al.*, 2008; Miles, 1999). Conversely, pectinases and polygalacturonases also produce breakdown products that may act as elicitors of reactive oxygen species (ROS) during plant defense (Smith and Boyko, 2007).

Plants rapidly activate callose deposition and the coagulation of proteins in the phloem to occlude compromised sieve elements, perceived through membrane depolarization and Ca^{2+} influx (Giordanengo *et al.*, 2010; Will and Van Bel, 2006). In turn, aphid saliva contains Ca^{2+}-chelating proteins that prevent the influx of calcium required to initiate a stress signaling cascade (Will *et al.*, 2007). A homolog of the calcium-binding protein regucalcin was recently discovered in the saliva of greenbug, (*Schizaphis graminum* Rondani) (Carolan *et al.*, 2009). Most strikingly, salivary proteins collected from artificial diets have been used to study the effect of aphid saliva on the dispersal of spindle-shaped forisomes, which are Ca^{2+}-sensitive phloem-specific contractile protein bodies currently only described in fabaceous species (Will *et al.*, 2007). The ability to sequester calcium likely helps not only to prevent sieve tube plugging and coagulation of phloem proteins in the food canal, but also to suppress the initiation of plant defense responses. It is tempting to draw analogies between the anticoagulant properties of aphid saliva and that of hematophagous hemipterans such as bed bugs (Cimicidae).

Reports on the number of proteins contained in aphid saliva vary by species, collection method and sensitivity of detection technique. Saliva from the pea aphid (*Acyrthosiphon pisum* Harris) yielded only nine proteins that could be resolved using 1D-electrophoresis (Carolan *et al.*, 2009), whereas vetch aphid (*Megoura viciae* Buckton) saliva yielded 29 proteins in a similar study (Will *et al.*, 2007). A bioinformatics approach based on the presence of secretion signals in salivary gland EST data from green peach aphid (*Myzus persicae* Sulzer) predicted 115 secreted proteins (Bos *et al.*, 2010). The functions of the majority of salivary proteins are unknown. In fact, many proteins discovered in aphid saliva do not exhibit significant homology to any known protein listed in sequence databases. Out of more than 200 peptides identified from the salivary proteome of *M. persicae* using 2D-electrophoresis coupled to mass spectrometry (MS), only 71 matched known sequences or functions (Harmel *et al.*,

2008). However, some of the data reported in the Harmel *et al.* study is dubious as out-of-frame matches to the *M. persicae* EST database were reported and some peptides were non-tryptic. Furthermore, the alkylation chemistry performed on the samples using iodioacetamide was insufficient to block reduced cysteines and the excess of dithiothreitol the authors used for reduction. The Harmel *et al.* study demonstrates the urgent need for a very careful review of the entomology proteomics literature that is published in non-proteomics journals.

Half of the more than 300 proteins from the *A. pisum* salivary gland proteome detected in a recent study have no homology to any known protein, a much higher percentage than the average calculated for the entire *A. pisum* genome (Carolan *et al.*, 2011). These novel proteins likely have critical functions in aphid survival and virulence. An EST library prepared from the salivary gland of *A. pisum* yielded a highly abundant transcript designated *Coo2*, which encodes a protein of unknown function; when silenced via siRNA, reduced levels of this transcript severely reduced the lifespan of adult aphids (Mutti *et al.*, 2006) and hindered the establishment of a successful feeding site (Harmel *et al.*, 2008). Conversely, *Coo2* also enhances the fecundity of *M. persicae* when overexpressed in *Nicotiana benthamiana* leaf discs (Bos *et al.*, 2010). Another aphid protein was also recently identified as a potential effector protein: Mp10, a protein from *M. persicae* saliva, causes chlorosis when overexpressed in tobacco leaves (Bos *et al.*, 2010).

It is clear that plants also use salivary compounds to detect aphid presence and elicit resistance responses, since *M. persicae* aphids feeding on *Arabidopsis* plants infiltrated with salivary proteins exhibited reduced fecundity compared to control plants (Bos *et al.*, 2010; De Vos and Jander, 2009). We know from ammonium sulfate-precipitated extracts injected into resistant wheat that the Russian wheat aphid (*Diuraphis noxia* Kurdjumov) elicitor(s) are proteins (Lapitan *et al.*, 2007). Similar

experiments with fractionated saliva from *M. persicae* infiltrated into *Arabidopsis* suggested the existence of a protein elicitor less that 10 kDa in size (De Vos and Jander, 2009). No aphid elicitor, acting as an aphid-derived *avr* gene product interacting with a plant *R* gene product to induce aphid resistance, has been described to date.

The Russian wheat aphid salivary secretome

Feeding by *D. noxia* causes severe toxicosis in susceptible cereal crops, which manifests as leaf rolling, longitudinal chlorotic streaking, stunting and the eventual collapse of the plant (Burd and Burton, 1992). This is in contrast to the interactions of most aphid species and their plant hosts, which do not cause such dramatic changes in plant phenotype (Miles, 1999). Fractionated extracts from *D. noxia* injected into susceptible wheat cause the same dramatic symptoms, but differ between extracts sourced from different biotypes (Lapitan *et al.*, 2007). The prevalence of new biotypes is of major concern to plant breeders developing new resistant wheat varieties (Randolph *et al.*, 2009). A new biotype called RWA2 was first detected in Colorado in 2003 (Haley *et al.*, 2004). This had serious consequences for the wheat industry, since most resistant cultivars deployed at that time relied on a single resistance gene, *Dn4*, and an urgent collaborative effort was initiated to find accessions genetically resistant to RWA2 infestation and employ that resistance in breeding programs (Collins *et al.*, 2005). To date, eight biotypes have been identified in the United States (Liu *et al.*, 2010), with five of those occurring in Colorado (Weiland *et al.*, 2008).

New aphid biotypes are assigned based on the phenotypic responses of plants, not on genetic differences between aphids (Burd *et al.*, 2006). There have been several reports of differential wheat gene expression in response to different *D. noxia* biotypes (Botha *et al.*, 2010; Lapitan *et al.*, 2008; Zaayman *et al.*, 2009). Although genetic variation between *D. noxia* populations has been assayed (Liu *et al.*, 2010), how those

genetic differences correlate to changes in virulence is not currently known. We wanted to identify differentially expressed proteins from the secreted salivary proteome of two different *D. noxia* biotypes. Since we know that the agents responsible for eliciting defense responses in wheat are proteins (Lapitan *et al.*, 2007), we hypothesize that qualitative or quantitative differences in protein content of the saliva are responsible for observed differences in virulence. Such proteins are probably secreted into the plant to interact with plant proteins and modulate their function.

There are several advantages to employing a proteomics approach instead of assaying these differences on the transcriptomic level. Collecting the proteins from aphid saliva not only allows for the reduction of sample complexity to a manageable set of gene products, but also narrows down the complexity to only that which is biologically relevant: in this case, proteins that are actually secreted into the plant tissue. In a recent study using publicly available EST data, 19 out of 134 *M. persicae* salivary proteins predicted to have signal peptides also had transmembrane domains, indicating that they would likely remain embedded in the membrane upon secretion (Bos *et al.*, 2010). It is also possible that some secreted proteins lack a signal peptide, or are produced elsewhere in the aphid and passed to the salivary glands via the hemolymph, implying that neither the transcripts nor the protein would necessarily show increased abundance in salivary gland tissue (Carolan *et al.*, 2011). Collecting proteins from aphid saliva secreted into a sterile diet is a viable technique that has been reported by several research groups (Carolan *et al.*, 2009; Cherqui and Tjallingii, 2000; Cooper *et al.*, 2011; De Vos and Jander, 2009; Harmel *et al.*, 2008). Previous artificial feeding studies indicate that more total soluble protein could be collected from a sucrose diet, compared to an amino acid or water diet (Cooper *et al.*, 2010). However, protein yield from diet-collected saliva remains a hindrance to aphid secretome characterization. De Vos and Jander (2009) determined that a protein or proteins less than 10 kDa in size were

responsible for inducing resistance to *M. persicae* in *Arabidopsis*. However, they were unable to visualize any proteins smaller than 10 kDa using SDS-PAGE. In this case, shotgun mass spectrometry is a much more sensitive approach that does not require a large yield of protein from an excised gel spot for peptide analysis.

DIGE, iTRAQ and MuDPIT as proteomics approaches

We elected to use a combination of proteomics methods to analyze the *D. noxia* salivary proteome. These included conventional 2D-electrophoresis, fluorescence differential gel electrophoresis (DIGE) (Alban *et al.*, 2003) coupled to LC-MS-MS/MS analysis of excised protein spots, and shotgun LC-MS-MS/MS to identify proteins secreted in *D. noxia* saliva. We subsequently used a combination of iTRAQ (Ross *et al.*, 2004) and MuDPIT analysis (Link *et al.*, 1999) to detect peptides derived from proteins of lower abundance and accurately quantify any differences in protein abundance between RWA1 and RWA2.

Fluorescent DIGE is a multiplexing 2D-electrophoresis technique based on the differential labeling of samples using Cy3 and Cy5 cyanine dyes (Ünlü *et al.*, 1997). Since the CyDyes are size and charge matched, identical proteins from both samples will co-migrate during electrophoresis. Co-migration on a single gel greatly improves reproducibility and allows for more accurate protein quantitation (Alban *et al.*, 2003). DIGE has been successfully used for aphid proteomics before (Cilia *et al.*, 2009; Cilia *et al.*, 2011a; Cilia *et al.*, 2011b; Francis *et al.*, 2010; Yang *et al.*, 2008).

Isobaric tags for relative and absolute quantification (iTRAQ) relies on a similar quantitative strategy, but allows for MS-based peptide quantification. Briefly, proteins are hydrolyzed, and the peptides from each sample labeled with isobaric amine tags. Samples are pooled and then analyzed by tandem mass spectrometry. In MS mode, differentially labeled peptides show no difference, but after fragmentation, reporter ions

of low molecular mass are generated, which are specific to each iTRAQ tag. This allows for the direct, quantitative comparison of peak signal intensities generated for identical peptides from different samples. iTRAQ also allows for multiplexing and the use of internal standards.

Multidimensional protein identification technology (MuDPIT) relies on two orthogonal separation systems, strong cation exchange (SCX) and reversed phase (RP), and eluted peptides are detected on a tandem mass spectrometer (Link *et al.*, 1999; Washburn *et al.*, 2001). This approach enables the analysis of highly complex protein samples. Most MS data is collected via data-dependent acquisition (DDA). In DDA mode, the mass spectrometer selects the most abundant precursor ions for MS/MS fragmentation that will break the peptide backbone and produce the series of ions in a spectrum to determine the sequence of amino acids. Since peptides from the most abundant proteins will produce the most abundant ions, DDA methods are irreproducible and only detect peptides derived from the most abundant proteins in the sample (Wolf-Yadlin *et al.*, 2007). MuDPIT helps to overcome this inherent limitation by fractionating the samples to increase the chromatographic resolution of the sample. This enables deeper sampling and more comprehensive coverage of the proteome (Link *et al.*, 1999). Online MuDPIT enhances the sensitivity of the assay because the first and second dimension separations are performed in tandem using the binary pump of the nanoHPLC to deliver the solvents for SCX and RP in a series of step-wise injections. This minimizes sample loss and enhances delivery of the sample onto the analytical column.

MATERIALS AND METHODS

Salivary gland isolation protocol development

A pilot study was conducted to determine whether salivary glands could be excised from Russian wheat aphids. A single adult aphid at a time was collected from a host plant with a paintbrush and placed on a clean glass slide under a stereomicroscope (Figure 4.1a). Using a razorblade, a quick and clean incision was made posterior to the prothorax to sever the head. A drop of phosphate buffered saline (PBS) was added to prevent desiccation of the tissues. With the head arranged with the ventral side facing upward, two microdissection needles were inserted directly above the labrum and moved apart to split the exoskeleton and expose the soft tissues within (Figure 4.1b). The microdissection needles were used to carefully clean away all of the dark chitinous exoskeleton. The salivary glands may still be attached to the brain through neural innervations and via ducts to the stylet, and these are carefully severed (Figure 4.1c). Isolated salivary glands (Figure 4.1d) were pushed to the edge of the PBS drop, allowing them to adhere to the tip of the needle. Glands are floated off into a tube containing 50 µl of RNA*later* tissue stabilization reagent (Ambion) until sufficient amounts have been collected for downstream analysis.

Aphid saliva collection

Two biotypes of Russian wheat aphid, RWA1 and RWA2, were housed in feeding cages and fed an artificial diet, from which their salivary proteins were recovered. Both biotypes were reared on susceptible wheat (*Triticum aestivum* L.) cv. 'TAM 107' for several generations prior to being moved onto the artificial diet. Feeding cages were constructed according to the method of Cooper *et al.* (2010). Working inside a sterile laminar flow bench, Parafilm (Pechiney Plastic Packagin, Chicago, IL) was stretched thinly across the bottom of a sterile 60 mm petri dish to form a sachet, into which was

pipetted 1.2 mL of sterile 15% sucrose (pH 7.2), before stretching it tight and sealing it. A 50 mm nitrile butadiene O-ring was placed on top of the sachet and covered by the petri dish lid, forming a secure crawlspace for the aphids. Each cage housed ~500 apterous adults and was incubated at 22 °C under a yellow light set to a 14:10 L:D photoperiod. The entire setup was replicated for a total of three separate biological repeats, with 130 cages constructed per biotype per replicate. Feeding cages incubated without aphids served as contamination controls. After 48 h of feeding, aphids were removed with a paintbrush, and the surface of the feeding sachet rinsed with sterile distilled water. Samples were collected by making an incision in the Parafilm at the edge of the dish, and aspirating the diet with a pipette. The underside of the Parafilm was rinsed by pipetting the diet up and down a few times to ensure the collection of any adhering proteins. Samples were collected into 50 mL centrifuge tubes and proteins precipitated from the artificial diet by the addition of 10% trichloroacetic acid, 60% acetone, 1% β-mercaptoethanol and incubating the samples at –20 °C overnight. Precipitated proteins were centrifuged at 5,000 *g* for 30 min at 4 °C, and the supernatant poured off. The walls of each 50 mL tube were washed extensively with ice-cold acetone to remove residual sucrose, and the sample repelleted by centrifugation at 7,000 *g* for 10 min at 4 °C. Samples were dried using a vacuum concentrator prior to solubilization in the appropriate solutions for downstream analysis.

Two-dimensional gel electrophoresis

A pilot study was conducted using two-dimensional gel electrophoresis to assess overall sample integrity from both aphid biotypes. Protein pellets were resuspended in 7M urea, 2M thiourea, 4% CHAPS (3-[(3-cholamidopropyl)dimethylammonio]-1-propane-sulfonate) overnight at 4°C with agitation. An analytical gel using fluorescence differential gel electrophoresis (DIGE) was run to enable quantitative comparisons

between the biotypes. Protein samples were labeled with CyDyes (GE Healthcare, Piscataway, NJ, USA) as follows: 10 μg of RWA1 with 80 pmoles Cy3 and 10 μg of RWA2 with 80 pmoles Cy5. Two individual preparative gels were run with 8 μg and 20 μg the remaining RWA1 and RWA2 protein samples respectively, and post-stained with SYPRO Ruby (Invitrogen, Carlsbad, CA, USA) overnight. First dimension isoelectric focusing (IEF) was carried out as follows: 13 cm pH 3–10NL IPG strips (GE Healthcare) were passively rehydrated overnight at RT. IEF was performed using the IpGphor II (GE Healthcare) using the following parameters: step: 500 V, 1 h 30 min; gradient: 1,000 V, 1 h; gradient: 8,000 V, 2 h 30 min; step: 8,000 V, remainder to 20.7 kVh. The second dimension SDS-PAGE was carried out using hand-cast 12% PAGE gels. Gels were run using a Hoefer SE 600 Ruby electrophoresis unit (GE Healthcare) for 4 h 30 min according to the following program: Step 1, 10 mA per gel; Step 2, 20 mA per gel. DIGE gels were scanned on the Typhoon 9400 Variable Mode Imager (GE Healthcare) after 2nd dimension separation. The SYPRO Ruby-stained gels were scanned on the Typhoon using a 610BP30 filter and 488 nm laser.

In-gel trypsin digestion

In total, 28 protein spots were excised from the SYPRO Ruby-stained RWA1 and RWA2 2D gels. 2D spots were digested using RapiGest surfactant (Waters, Milford, MA, USA) and trypsin (Promega, Wisconsin, WI, USA). Gel pieces were washed in series with: nano pure water, 50% acetonitrile (ACN)/50% 100 mM triethylammonium bicarbonate (TEAB), and ACN. Gel pieces were allowed to air dry and were then rehydrated in a solution of 0.1% RapiGest/50 mM TEAB for 10 minutes at 37 ° C. The excess solution was removed from the gel pieces and they were air dried briefly. A solution of 5 ng μL^{-1} sequence grade modified trypsin (Promega) in 50 mM TEAB was added to cover the gel pieces. Gel pieces were incubated overnight for digestion at 32 °C. Peptides were

extracted in a series of washes including: 50% ACN/2.5% formic acid (FA), 90 % ACN/0.1 % FA. To hydrolyze the RapiGest from peptide solutions, the pH was lowered to less than 3 using FA, and samples were incubated at 37 °C for 45 minutes. Samples were then centrifuged at 15,000 *g* for 10 minutes and the peptide supernatant transferred to new tubes and dried by vacuum centrifugation.

Mass spectrometry

Dried peptides were reconstituted with 12 µL of 3% ACN with 0.1% trifluoroacetic acid (TFA). Nano-LC separation of tryptic peptides was performed with a nanoACQUITY system (Waters), equipped with a Symmetry C18 5 µm, 20 mm x 180 µm trapping column and a UPLC BEH C18 1.7 µm, 15 cm x 75 µm analytical column (Waters). The samples, 5 µL partial loop injection, were transferred to the trapping column with a 0.1% solution of FA in water at a flow rate of 7 µL min^{-1} for 3 min. Mobile phase A consisted of 0.1% FA in water and mobile phase B consisted of 0.1% FA in ACN. Following desalting and concentration, the trapping column was subjected to a reverse flush to the analytical column and separated with a gradient of 2–40% mobile phase B over 30 minutes at a flow rate of 300 nL min^{-1}, followed by a 5 min rinse with 95% of mobile phase B. The column was re-equilibrated at initial conditions for 20 min. Column temperature was maintained at 35 °C. 100 fmol μL^{-1} [Glu^1]-fibrinopeptide B in 25% ACN with 0.1% FA was used as the lock mass compound and was delivered via the auxiliary pump of the LC system at a flow rate of 300 nL min^{-1} to the reference sprayer of the NanoLockSpray source of the mass spectrometer. The eluent from the analytical column was delivered to the analytical sprayer of the same source through a PicoTip emitter (New Objective, Woburn, MA, USA) with 10 µm tip diameter.

In-solution digestions

An aliquot of RWA2 salivary proteins was subjected to an in-solution digestion. The salivary pellets were resuspended in 0.1% RapiGest/50 mM TEAB solution at 4 ° C overnight. A Bradford assay was done to determine the protein concentration per sample. Approximately 50 µg of each sample was reduced in 5mM DTT, in 0.1% RapiGest/ 50 mM TEAB, in a starting volume of 50 µL; incubated at 50 °C for 30 minutes. Alkylation was carried out in 15 mM final iodoacetamide, in the dark, at RT for 30 minutes. 1 µg of trypsin was added to each sample for digestion at 37 °C for 3.5 hours. Following digestion, the RapiGest was hydrolyzed from the samples by decreasing the pH to less than 3 using TFA, and incubating at 37 °C for 45 minutes. Samples were then centrifuged at 15,000 *g* for 10 minutes, and the supernatant transferred to new tubes.

MS of Gel Spots

Mass spectrometric analysis of tryptic peptides was performed using a Synapt HDMS mass spectrometer (Waters, Milford, MA, USA). The Synapt was operated in Q-TOF V mode with a typical resolution of at least 10,000 FWHM. Analysis was conducted using positive polarity. The TOF analyzer of the mass spectrometer was externally calibrated using fragmentation of the doubly protonated monoisotopic ion of [Glu^1]-fibrinopeptide B delivered via the lock mass reference sprayer. Calibration was performed over the m/z range from 50–2,000. Collected data were post-acquisition lock mass corrected using the same [Glu^1]-fibrinopeptide B ion. The reference sprayer was sampled every 30 s for 1 s. Accurate mass LC-MS/MS data dependent acquisition (DDA) data were obtained as follows: MS survey scans of 1 s duration with an interscan delay of 0.02 s were acquired for the m/z range from 300–1,500. Charge state selection was enabled such that MS/MS data were obtained for up to three ions of charge 2+, 3+, or 4+ detected in the survey scans. MS/MS spectra were acquired for the m/z range from 50–2,000 at a scan rate of 1

s with an interscan delay of 0.02 s. Charge state-dependent collision energy ramps were employed to improve the quality of MS/MS spectra. A real time dynamic exclusion window of 40 s was applied to each precursor selected for fragmentation. The acquisition mode was switched from MS to MS/MS when the abundance of an individual ion exceeded 25 counts per second (cps), and returned to MS mode when the total ion current for the MS/MS acquisition exceeded 10,000 cps or after 3 scans had been completed. ProteinLynx Global Server 2.4 was used to process all raw data files into a .pkl peak list format compatible with MASCOT (Perkins *et al.*, 1999). The processing method components (*i.e.* mass accuracy, noise reduction, and deisotoping and centroiding) utilized identical processing criteria for electrospray survey and MS/MS functions as follows: lockmass correction was achieved using the doubly-protonated, monoisotopic peak for [Glu^1]-fibrinopeptide B at m/z 785.8426, using 2 lockspray scans with a tolerance of 0.25 Da. Noise reduction was achieved using adaptive background subtraction. Deisotoping and centroiding were done at the "medium" setting with automatic thresholds.

MS of in-solution digests

The RWA2 protein samples were submitted for shotgun LC-MS/MS analysis. These samples were reconstituted with 12 µL of 3% ACN with 0.1% TFA. The initial analysis was conducted as previously described for the 2D Gel Spot analysis with the following modifications. 1 µL injection volume was used. The gradient was modified from 2 – 40% B over 30 min to the same gradient over a 120 min period. Selection of up to 4 multiply charged (2^+–4^+) precursors per survey scan was allowed. For each precursor fragmented in MS/MS mode, analysis stopped after the TIC exceeded 10,000 cps or after 2.5 s had elapsed. Each precursor was written to a real time dynamic exclusion list for 120 s.

iTRAQ labeling

Three biological replicates of the two biotypes RWA1 and RWA2 were labeled as follows: RWA1_A, RWA1_B, RWA1_C; RWA2_A, RWA2_B, RWA2_C. Protein pellets were solubilized in 0.1% ProteaseMax (PMAX) surfactant (Promega), 50 mM TEAB. A Bradford assay was done to determine protein concentration. Samples were each aliquoted at 1 μg μL^{-1} starting protein in a total volume of 65 μL. For reduction and cysteine blocking steps, 50 mM TEAB and 0.1 % PMAX was added to the solution. Samples were reduced with 5 mM tris-(2-carboxyethyl)-phosphine (TCEP) for 20 min at 55 °C. To block the cysteines, 10 mM methyl-methane-thiosulfonate (MMTS) was added to each sample and incubated for 20 minutes at RT. A methanol/chloroform precipitation was carried out by adding the following to each sample: 4× volume methanol; 1× volume chloroform; 3× volume deionized water. The samples were left to precipitate at 4 °C for 1 h 15 min. The tubes were centrifuged at 15,000 *g* for 5 minutes at 4 °C. The supernatant was carefully removed from the protein pellet. Methanol was added to each pellet as 3× the sample volume, the pellets were gently vortexed, and centrifuged at 4 °C for 5 minutes. The methanol was removed carefully and the pellet was air dried briefly. The pellets were solubilized in 0.1 % PMAX/50 mM TEAB. One microgram of sequence grade modified trypsin was added and samples were incubated overnight at 37 °C. The digests were put into a vacuum centrifuge until almost dried. 30 μL of 500 mM TEAB was added to each sample. The iTRAQ labeling reagents were each reconstituted in 50 μL of isopropanol. The entire iTRAQ label volume was added to the appropriate sample. The samples were incubated at RT for 2 h during the labeling reaction. TFA was added to each sample to bring the pH below 4. Labeled samples were then pooled to one single tube and mixed for analysis.

Protein identification

The MS and MS/MS data collected were submitted to Mascot v.2.3 (Matrix Science, Boston, MA) using an in-house MASCOT server for database interrogation. The experimental data were searched against the entire NCBI non-redundant database containing the *A. pisum* gene models (download date: January 2011) or a database of *S. graminum* 454 short sequence reads (Cilia et al, unpublished). The following search parameters were used: carbamidomethyl-cysteine as a fixed modification, methionine oxidation as a variable modification, and one missed tryptic cleavage. The searches were done with a mass error tolerance of 25 ppm in the MS mode and 0.1 Da in the MS/MS mode. The preliminary protein identifications obtained automatically from the software were inspected manually for peptides containing significant (E value < 0.05) matches prior to acceptance. False discovery rate (FDR) was 0% at $P < 0.05$ in all searches.

RESULTS

Salivary gland isolation protocol development

To our knowledge, the successful isolation of intact salivary glands from *D. noxia* has not been reported before. We attempted this procedure while exploring methods for obtaining samples enriched for salivary transcripts or proteins for downstream analysis (Figure 4.1). Although a time-consuming process, intact principal and accessory glands were successfully isolated (Figure 4.1d). Preliminary RT-qPCR analysis (data not shown) indicated that the samples were highly enriched for *Coo2*, a salivary gland-specific transcript (Mutti *et al.*, 2008).

2D-electrophoresis of secreted Russian wheat aphid salivary proteins

We estimate that salivary proteins were collected from a total of more than 500,000 aphids. Average protein yield was 100 µg per biotype per replicate. We observed no obvious differences in settling time or the amount of time spent in a stationary feeding position between RWA1 and RWA2. Unprobed diets showed no signs of microbial growth, and no protein could be isolated from them. Our trial DIGE images suggested that the RWA1 and RWA2 secretomes are highly similar, with only a few protein spots unique to each sample (Figure 4.3). To ascribe putative identities to some of the pertinent and differentially expressed protein spots, we resolved RWA2 (Figure 4.4) and RWA1 (Figure 4.5) protein samples on individual 2D gels, and excised 28 gel spots of interest. Spots were subjected to in-gel digestion and nano-LC-MS-MS/MS analysis and mass spectra were searched against an in-house MASCOT database. Significant protein similarities from gel spots are summarized in Table 4.1, and include two spots with similarity to a cuticular protein, two spots with similarity to a glucose dehydrogenase-type oxidoreductase, and two spots with similarity to actin related protein 1. We also performed shotgun nano-LC-MS-MS/MS analysis on an in-solution digest of RWA2 secreted salivary proteins to discover proteins that could not be obtained from excised gel spots. The shotgun results are summarized in Table 4.2 and also suggest the presence of glucose dehydrogenase in secreted *D. noxia* saliva. Additional proteins found include an aminopeptidase, apolipophorin and two proteins of unknown function.

iTRAQ and MuDPIT analysis

Out of a total of 1,460 proteins identified using this approach, six aphid-specific protein matches were discovered (summarized in Table 4.3). These include the glucose dehydrogenases and aminopeptidases observed using the 2D-electrophoresis and

shotgun MS approaches, and also a number of proteins of unknown function. BLAST searches using accession gi|193676365 resulted in significant alignment of the C-terminal with glucose dehydrogenases from other insects. However, the N-terminal of this protein appears to be unique: the Phyre2 protein fold recognition server (Kelley and Sternberg, 2009) was used to model a predicted structure for the 500 residues at the N-terminal, but produced no significant alignments. BLAST searches using accession gi|328713643 produced alignments to insect lipid-binding proteins, including apolipophorin and vitellogenin. A predicted structure was modeled for this protein using Phyre2: 73% of the N-terminal 1200 amino acids were modeled to the top scoring protein structure, lipovitellin, with 100% confidence. BLAST searches for accession gi|328713749 produced alignments to SD repeat proteins, including bacterial flagellar proteins. Accession gi|328724556 produced alignments to various microbial proteins, including viral type A inclusion protein. Accession gi|193580006 produced no significant BLAST alignments to any protein in NCBI. Interestingly, iTRAQ reporter ions for accession gi|193659688 were only detected in RWA1, and not RWA2 samples. BLAST searches for accession gi|328705553 produced weak alignments to SD repeat proteins.

DISCUSSION

Approaches to aphid saliva sample preparation

cDNA prepared from isolated *D. noxia* salivary glands was highly enriched for *Coo2*, a salivary gland-specific transcript (Mutti *et al.*, 2008), indicating that intact salivary glands suitable for transcriptomics analysis can be isolated using the method we developed. We managed to obtain both principal and accessory salivary glands (Figure 4.1d) from *D. noxia*. The larger principal glands mainly contribute to the gel-like saliva that forms the stylet sheath. Since the accessory glands are responsible for virus

transmission from aphids to plants, it is inferred that they are the source of E1 watery saliva (Tjallingii, 2006), although based on the innervation of the principal salivary glands, aphids might be able to adjust the composition of saliva. Although we collected whole salivary glands for our pilot study, it would be possible to collect principal and accessory salivary glands into separate pools for downstream analysis. Aphid salivary gland proteomes tend to be very complex, consisting of many different proteins. Not all of the proteins present in the salivary gland are likely to be secreted. To sift out the proteins most likely to be secreted, researchers using salivary glands as starting material have scrutinized EST and/or protein sequences for secretion signals (Bos *et al.*, 2010; Carolan *et al.*, 2011). The approach we elected to take is to collect proteins that are secreted by the aphid into an artificial diet, since it is likely that these proteins would also be secreted into sieve elements. The characterization of secreted salivary proteomes obtained from artificial diets has been reported for several aphid species, including *A. pisum* (Carolan *et al.*, 2009; Cooper *et al.*, 2011), *M. persicae* (Harmel *et al.*, 2008), *S. graminum* (Cherqui and Tjallingii, 2000; Ma *et al.*, 2010), and *D. noxia* (Cooper *et al.*, 2010). However, low protein yield from artificial diets has been a major impediment to comprehensive characterization of secreted proteomes, which is why we appreciably scaled up the amount of feeding cages and total amount of aphids employed in sample collection. Several salivary protein purification approaches have been published, including phenol, multi-detergent, and TCA-acetone extraction methods (Cilia *et al.*, 2009; Harmel *et al.*, 2008), or concentrating samples using dialysis spin columns with various molecular weight cut-offs (Carolan *et al.*, 2009; Cooper *et al.*, 2010). However, since Cilia *et al.* (2009) reported the most reproducible results and the highest protein yield from TCA-acetone extraction, we selected this method for preparing our *D. noxia* protein samples.

Comparison of Russian wheat aphid biotype secreted salivary proteomes using DIGE

The majority of protein spots visualized via DIGE were of high molecular weight, which is similar to that reported by Carolan *et al.* (2011). We had many more spots of better intensity than previously reported from *D. noxia* salivary extracts (Figure 4.3), although previous reports used only ~3 µg of protein for 2D analysis (Cooper *et al.*, 2010, 2011). However, the sheer number of spots compared to previous reports indicated a high level of background, and we suspected this might be of microbial origin. We picked almost 30 protein spots from subsequent SYPRO Ruby-stained gels (Figure 4.4, 4.5) and assigned putative identities to these protein spots using LC-MS-MS/MS coupled with database homology searches. The majority of gel spots were either of microbial origin, or resulted in no significant database hits. This either reflects insufficient yield from the excised protein for accurate MS detection, or that these proteins have no homology to known proteins in the database.

Six protein spots had significant homology to aphid-specific peptides (Table 4.1). Both spot 14 and 16 were similar to glucose dehydrogenase, an oxidoreductase-type enzyme. Oxidoreductases are some of the many effectors present in the saliva of caterpillars (Thivierge *et al.*, 2010) and glucose dehydrogenase specifically has been previously reported from secreted *A. pisum* (Carolan *et al.*, 2009), *M. persicae* (Harmel *et al.*, 2008) and *D. noxia* (Cooper *et al.*, 2010, 2011) salivary proteomes. Glucose dehydrogenase has an important immunological role in the encapsulation of entomopathogenic fungi (Lee *et al.*, 2005). Its role as a component of secreted saliva is less clear, but it has been suggested that, as an oxidoreductase enzyme, it might play a role in the quenching of plant ROS responses (Carolan *et al.*, 2011).

To test whether the microbial background observed in our samples was introduced via laboratory technique, we set up non-sterile aphid feeding cages along with cages made using Parafilm soaked in 80% ethanol and dried in a laminar flow hood, and

subsequently UV-sterilized for an hour. Salivary proteins collected from these two sets of feeding cages were compared using in-solution digestions followed by label-free mass spectrometry. Results showed no difference between samples collected from sterile and non-sterile feeding cages, suggesting that the microbial proteins were introduced by the aphids themselves. This pilot study allowed us to construct an MS exclusion list to reduce the background from spectra derived from bacterial proteins.

Database searches using our shotgun MS data revealed additional aphid-specific proteins not identified from the 2D-gels (Table 4.2). These included accession gi|193594294, with significant homology to membrane alanyl aminopeptidase. This aminopeptidase is an M1 zinc-dependant metalloprotease also reported in salivary glands and secreted saliva of *A. pisum* (Carolan *et al.*, 2009; Carolan *et al.*, 2011). They are also components of the salivary secretome of the root-knot nematode (*Meloidogyne incognita* Kofoid and White) (Bellafiore *et al.*, 2008). It is hypothesized that aminopeptidases are either involved in digestive processes, or might degrade plant proteins that may be involved in defense (Carolan *et al.*, 2009).

Accession gi|193610805 exhibited high homology to apolipophorin. Insect apolipohorins are hemolymph proteins more generally associated with diglyceride transport, and endogenous apolipophorins bind to pathogen elicitor molecules whereupon they induce the insect innate immune response (Whitten *et al.*, 2004). Apolipophorin has been reported from the salivary glands of *A. pisum* (Carolan *et al.*, 2011) and another phloem-feeding hemipteran, the rice brown planthopper (*Nilaparvata lugens* Stål) (Konishi *et al.*, 2009). It is suggested that secreted apolipophorins could interfere with the signaling of the plant's own cellular immune response (Carolan *et al.*, 2011).

iTRAQ comparison of biotype secretomes using MuDPIT fractionation

Since microbial proteins were so highly abundant in our secreted salivary samples, we used MuDPIT as a highly-resolving MS approach to search for less-abundant aphid proteins. Although we identified a total of 1,460 proteins using this approach, only a small number of these had significant homology to aphid sequences in the databases (Table 4.3). Included in this list were the glucose dehydrogenases, aminopeptidases and apolipohorins discovered with the previous MS approaches, providing independent validation for the presence of these proteins in the secreted salivary proteome of *D. noxia*. Although we have employed some of the most advanced technology currently available to do comprehensive peptide discovery within our sample, the total number of aphid proteins identified in our studies remains small, which agrees with the secreted salivary proteomes previously reported (Cherqui and Tjallingii, 2000; Cooper *et al.*, 2010, 2011; Harmel *et al.*, 2008). These numbers do not match the large and diverse numbers of proteins reported from salivary gland proteomes (Bos *et al.*, 2010; Carolan *et al.*, 2011), even when limiting analysis to the fraction of proteins containing secretory signal peptides. There are several plausible explanations for this observation. Artificial diets do not closely mimic plant phloem sap, and may therefore not provide identical gustatory feedback to the aphid, causing a differential release of only a subset of proteins. There is also inherent variability between different protein extraction protocols, and the TCA-acetone protocol we used would not necessarily extract every possible protein found in aphid saliva (Cilia *et al.*, 2009). It is also entirely plausible that aphids simply do not secrete all the proteins with signal peptides found in salivary gland libraries. Such conjecture is warranted, based on the different uses of E1 and E2 salivation; salivary composition is likely different between E1 and E2 salivation. Since E2 salivation is sucked back up the food canal with ingested phloem sap, proteins secreted during E2 do not enter the plant cell. Therefore, not all secreted peptides enter the plant

cell, but might still interact with plant-derived molecules within the alimentary canal to prevent coagulation and supply gustatory feedback to the aphid.

Aphids of different species appear to have several salivary proteins in common, which are likely essential for establishing and maintaining a successful feeding site. Salivary gland proteomes are complex, but the secreted proteins detected using current technology and artificial diets are much less complex. Taken together, these lines of evidence suggest that proteomics using salivary glands as starting material might be overestimating the number of aphid proteins that are secreted into plants. This is useful for planning future research aimed at investigating plant–aphid protein interaction networks, or trying to uncover aphid proteins that either suppress or activate plant defense responses. It is also clear that simplified artificial diets do not provide the same gustatory feedback to aphids, and it is therefore possible that secretomes collected from them do not accurately reflect the composition of saliva secreted *in planta*. However, we did uncover a number of unusual proteins from secreted *D. noxia* saliva, which warrant further study as possible elicitors or effectors.

From the number of microbial proteins identified in artificial diet subjected to aphid feeding, we conclude that *D. noxia* might inoculate bacteria or bacterial proteins into plants, and that this might have a beneficial effect on aphid feeding and reproduction. Bacterial elicitors would induce an inappropriate salicylic acid (SA)-mediated senescence response, thereby mobilizing chloroplast nitrogen back into the phloem and nutritionally enriching the aphid food source. Since SA and jasmonic acid (JA) responses are antagonistic (Chisholm *et al.*, 2006), this would cause the downregulation of JA responses known to be effective against herbivores (Chen, 2008). This would also help explain why genes involved in both SA and JA signaling are differentially regulated during plant responses to aphids. How microbial populations differ between aphid biotypes is currently unknown, and will be the focus of future study.

Table 4.1. Protein identities for *D. noxia* proteins excised from 2D polyacrylamide gels

Spot number	Accession number[a]	Protein ID	Sequences	Protein Score	E-value	Peptide sequence
10	gi|193620175	PREDICTED: similar to cuticular protein 15 from Low Complexity family (AGAP009759-PA) [*Acyrthosiphon pisum*]	6	68.19	6.40E-07	VPYPVQVPVEVK
11	gi|193620175	PREDICTED: similar to cuticular protein 15 from Low Complexity family (AGAP009759-PA) [*Acyrthosiphon pisum*]	6	67.38	9.70E-07	VPYPVQVPVEVK
14	gi|193627355	PREDICTED: similar to glucose dehydrogenase, partial [*Acyrthosiphon pisum*]	2	57.38	3.50E-05	FLTEQEDNLFK
16	gi|193659536	PREDICTED: similar to AGAP003785-PA [*Acyrthosiphon pisum*]	7	85.62	3.50E-08	YTAQEDDTFGQGLENK
18	gi|217330650	actin related protein 1 [*Acyrthosiphon pisum*]	11	85.03	2.40E-08	AVFPSIVGRPR
25	gi|217330650	actin related protein 1 [*Acyrthosiphon pisum*]	7	90.39	1.20E-08	AGFAGDDAPR

[a]NCBI accession number in local database created on the Cornell University Mascot v. 2.3 server July 9th, 2011, all NCBI searched.
[b]Total number of unique peptides contributing to the score.

Table 4.2. Predicted peptide identities from in-solution digested *D. noxia* salivary proteins

Accession number [a]	Protein ID	Sequences [b]	Protein score	E-value	Peptide sequence
gi|193594294	PREDICTED: similar to membrane alanyl aminopeptidase N [Acyrthosiphon pisum]	1	56	5.40E-05	WLAVTQFEPTYAR
gi|193595360	PREDICTED: hypothetical protein [Acyrthosiphon pisum]	2	105	4.00E-06	VSTINLGVFDFNKK
gi|193610805	PREDICTED: similar to apolipophorin [Acyrthosiphon pisum]	2	112	1.60E-06	EGYLGVGALLR
gi|193627355	PREDICTED: similar to glucose dehydrogenase, partial [Acyrthosiphon pisum]	5	127	2.30E-06	FLTEQEDNLFK
gi|193659536	PREDICTED: similar to AGAP003785-PA [Acyrthosiphon pisum]	3	188	9.80E-10	YTAQEDDTFGQGLENKR
gi|193676365	PREDICTED: similar to GA21838-PA [Acyrthosiphon pisum]	5	108	2.70E-08	YMVSTTSSTAGSCR

[a]NCBI accession number in local database created on the Cornell University Mascot v. 2.3 server July 9th, 2011, all NCBI searched.
[b]Total number of unique peptides contributing to the score.

Table 4.3. iTRAQ comparison of secreted proteins from *D. noxia* biotypes RWA1 and RWA2.

Accession number[a]	Protein ID	MASCOT Score[b]	E-value[c]	Sequences[d]	Protein detected	
					RWA1	RWA2
gi|193676365	Hypothetical protein	754	1.2e-05	20(3)	+	+
gi|328709186	Glucose dehydrogenase (acceptor-like)	346	1.3e-02	4(4)	+	+
gi|193659536	Glucose dehydrogenase (acceptor-like)	912	2.5e-04	22(3)	+	+
gi|328713643	Hypothetical protein	205	8.7e-03	9(2)	+	+
gi|328719823	Aminopeptidase N-like	93	0.057	1(1)	+	+
gi|328713749	Hypothetical protein	87	0.05	1(1)	+	+
gi|328724556	Hypothetical protein	63	0.01	1(1)	+	+
gi|328709894	Aminopeptidase N-like	62	0.081	1(1)	+	+
gi|193580006	Hypothetical protein	59	0.078	2(1)	+	+
gi|193659688	S-adenosyl-L-homocysteine hydrolase	58	0.052	1(1)	+	–
gi|328705553	Hypothetical protein	50	0.2	1(1)	+	+
gi|193715980	Trehalase-like isoform 1	49	0.3	1(1)	+	+
gi|239789413	Carbonic anhydrase 7-like	45	0.22	1(1)	+	+

[a]NCBI accession number in local database created on the Cornell University Mascot v. 2.3 server July 9th, 2011, all NCBI searched.
[b]MASCOT score incorporating a probability-based algorithm into the MOWSE scoring algorithm (Pappin *et al.*, 1993). Score is calculated as follows: -10*LOG10(P), where P is the absolute probability that the observed match is a random event.
[c]Expect (E) value for highest scoring peptide contributing to the protein identification.
[d]Total number of unique peptides contributing to the score and in parenthesis, number of unique peptides with iTRAQ ratios contribution to quantification.
[e]Weighted ratios. For each reporter ion, the intensity values of the assigned peptides are summed and the protein ratio(s) are calculated from the summed values. If a ratio is shown in bold, it is significantly different from 1 at a 95% confidence level. Data were normalized using summed intensities. A correction factor was applied such that the sum of the intensities for a reporter ion peak over all peptide matches that pass the quality tests is the same for all the reporter ions.

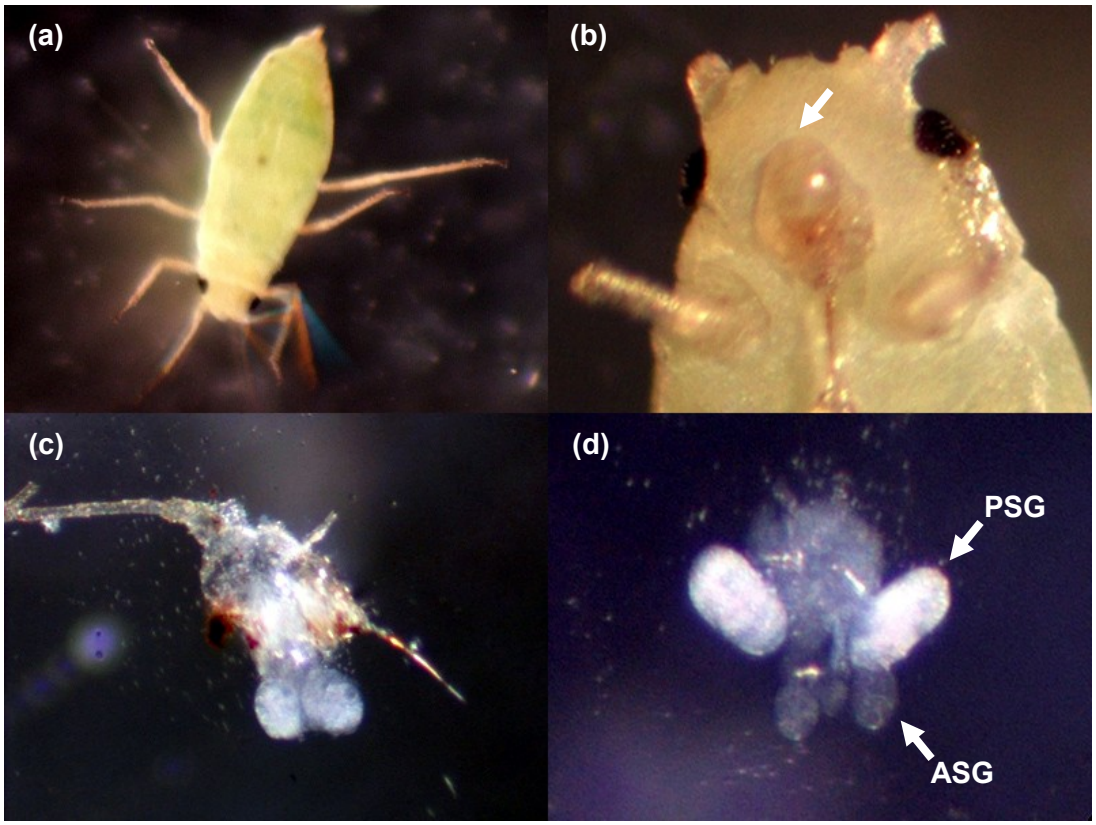

Figure 4.1. *Diuraphis noxia* salivary gland isolation. (a) Adult aphid on glass slide. (b) Ventral view of head. Point of insertion of microdissection needle indicated by arrow. (c) Salivary glands exposed. (d) Principal salivary glands (PSG) and accessory salivary glands (ASG), still connected to some brain tissue.

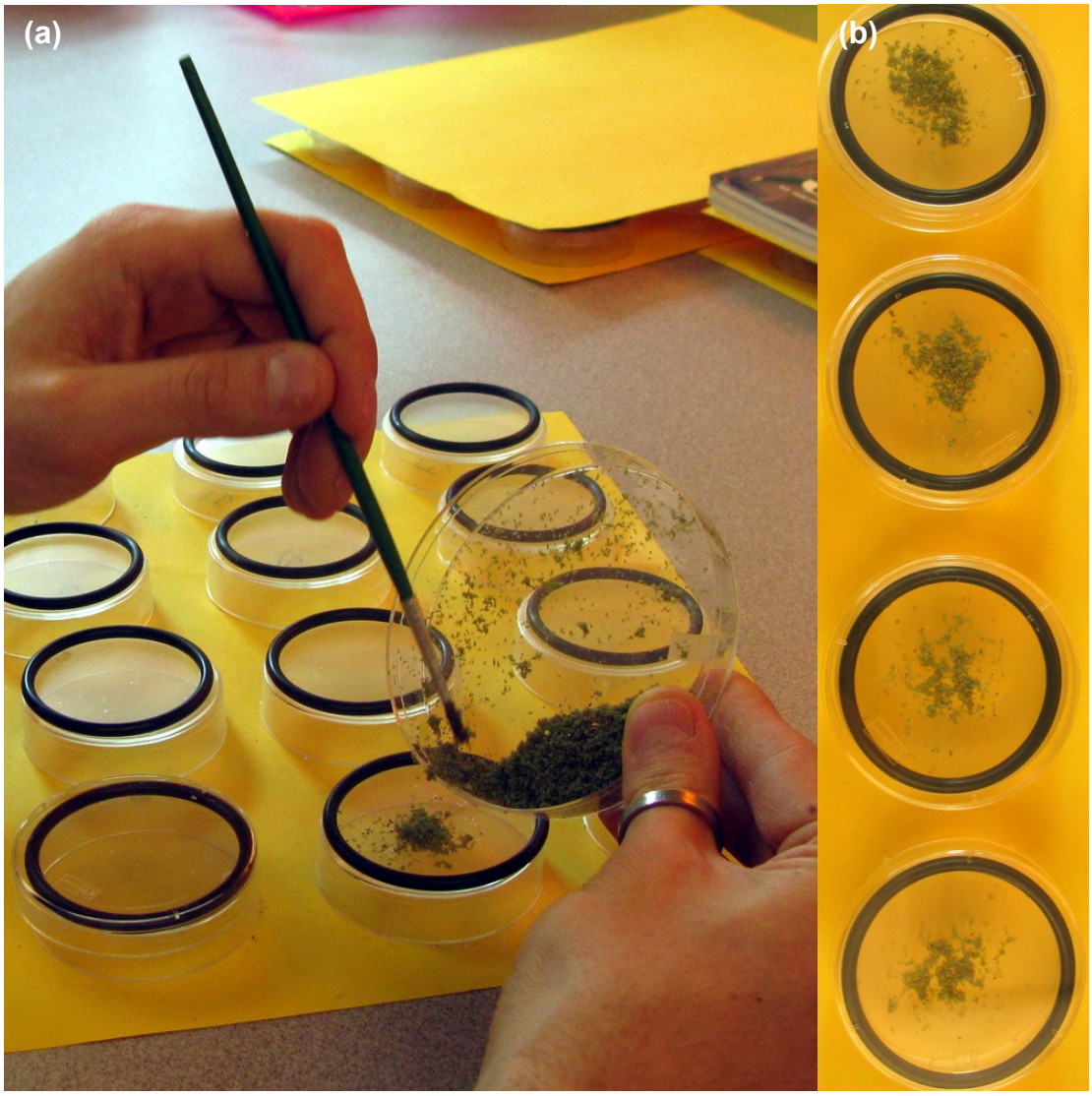

Figure 4.2. (a) Infestation of feeding cages constructed from petri dishes. A layer of Parafilm is stretched across the bottom of each petri dish, and a 15% sucrose artificial diet pipette into the space underneath, forming a feeding sachet. Aphids are placed on top and covered with the petri dish lid, supported by a rubber O-ring to provide a crawlspace. (b) Aphids were incubated in these cages under yellow light for 48 h before the diet was collected for salivary protein isolation.

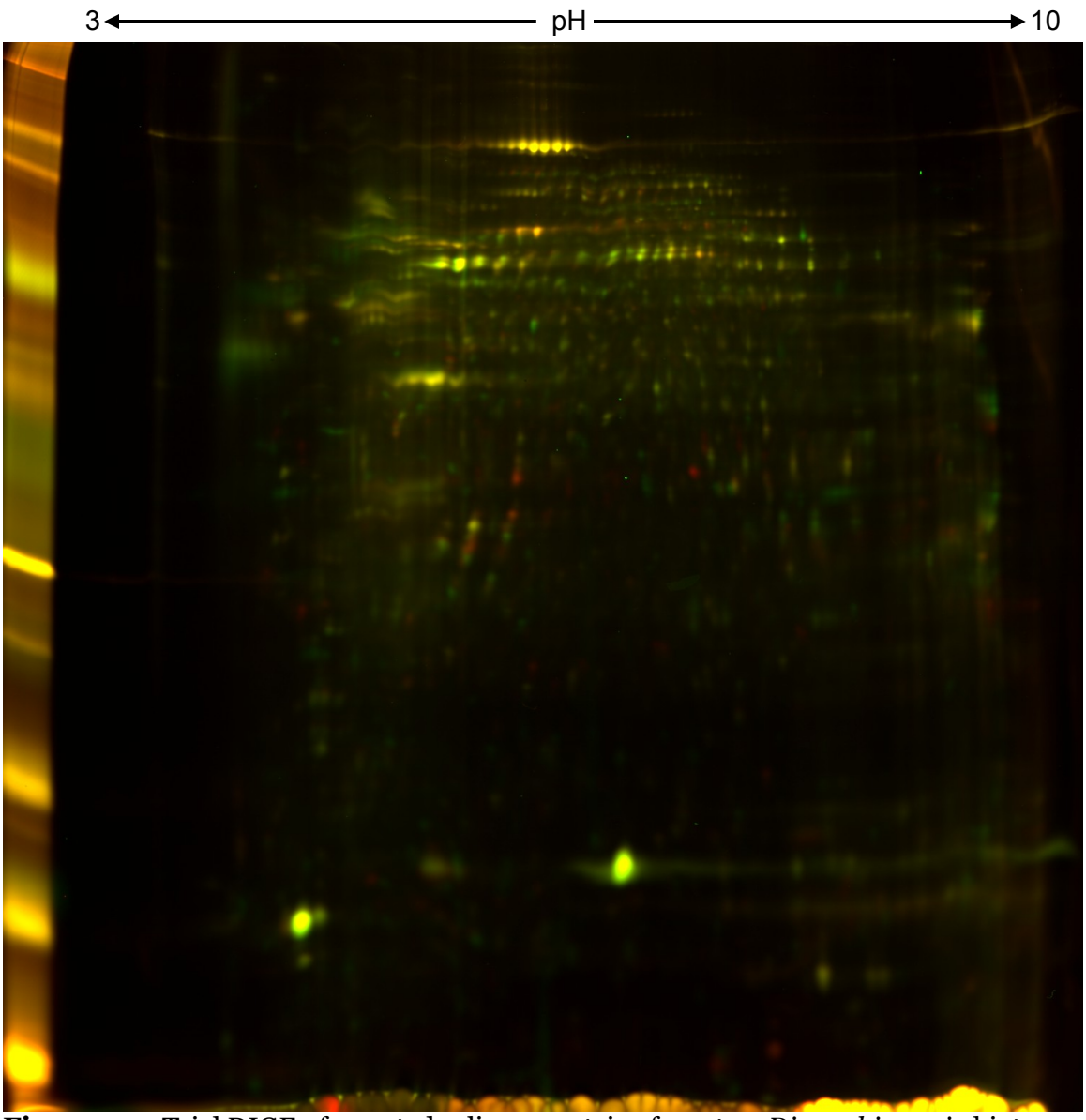

Figure 4.3. Trial DIGE of secreted salivary proteins from two *Diuraphis noxia* biotypes. Proteins were separated on a 3–10NL IPG strip in the first dimension, and on 12% SDS-PAGE in the second dimension. Proteins from RWA1 are labeled with Cy3 (green); proteins from RWA2 are labeled with Cy5 (red).

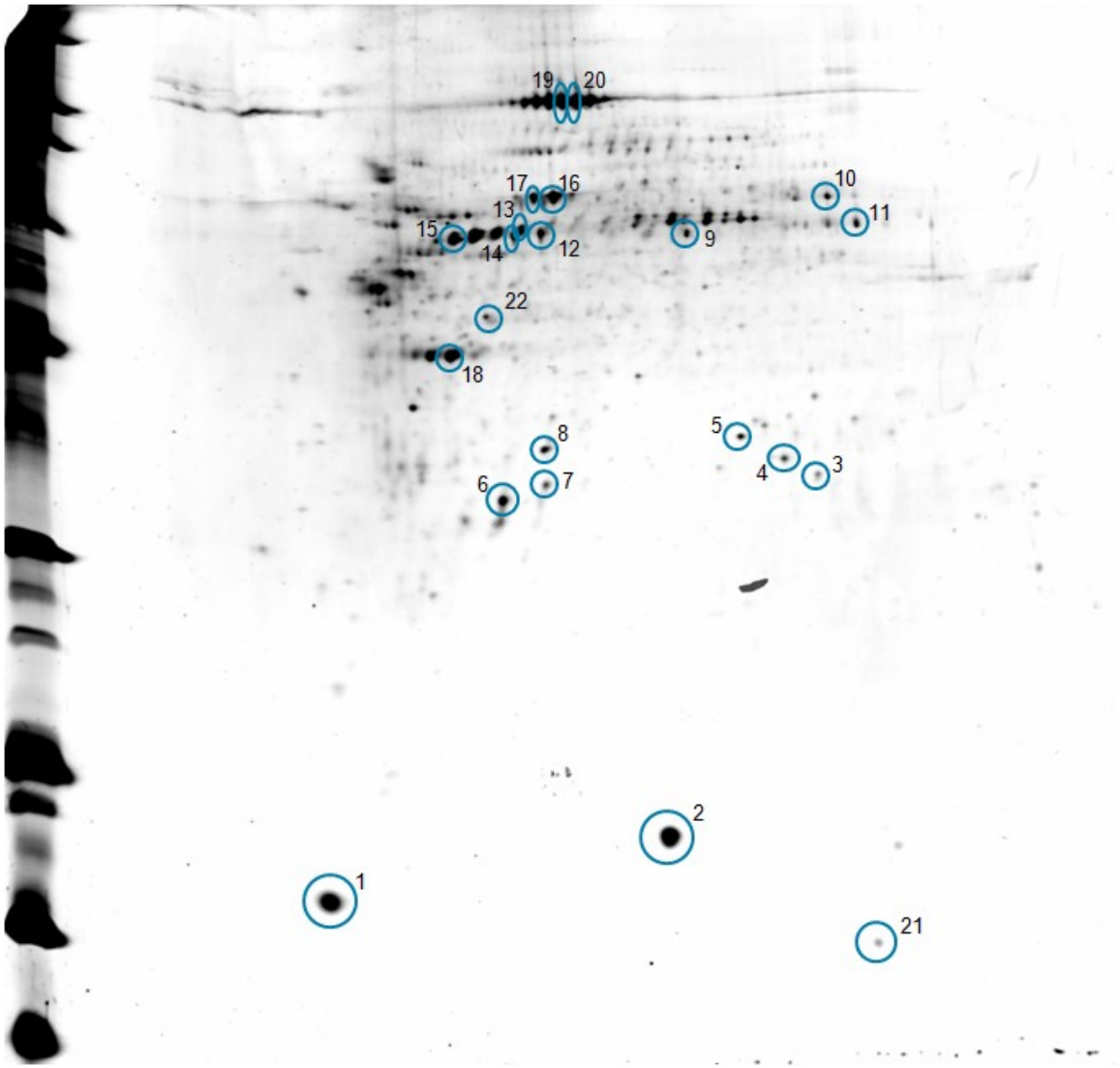

Figure 4.4. SYPRO Ruby stained secreted salivary proteome of the RWA2 biotype of *Diuraphis noxia*. A total of 20 μg of protein was separated on a 3–10NL IPG strip in the first dimension, and on 12% SDS-PAGE in the second dimension. Spots were excised and subjected to nano-LC-MS-MS/MS.

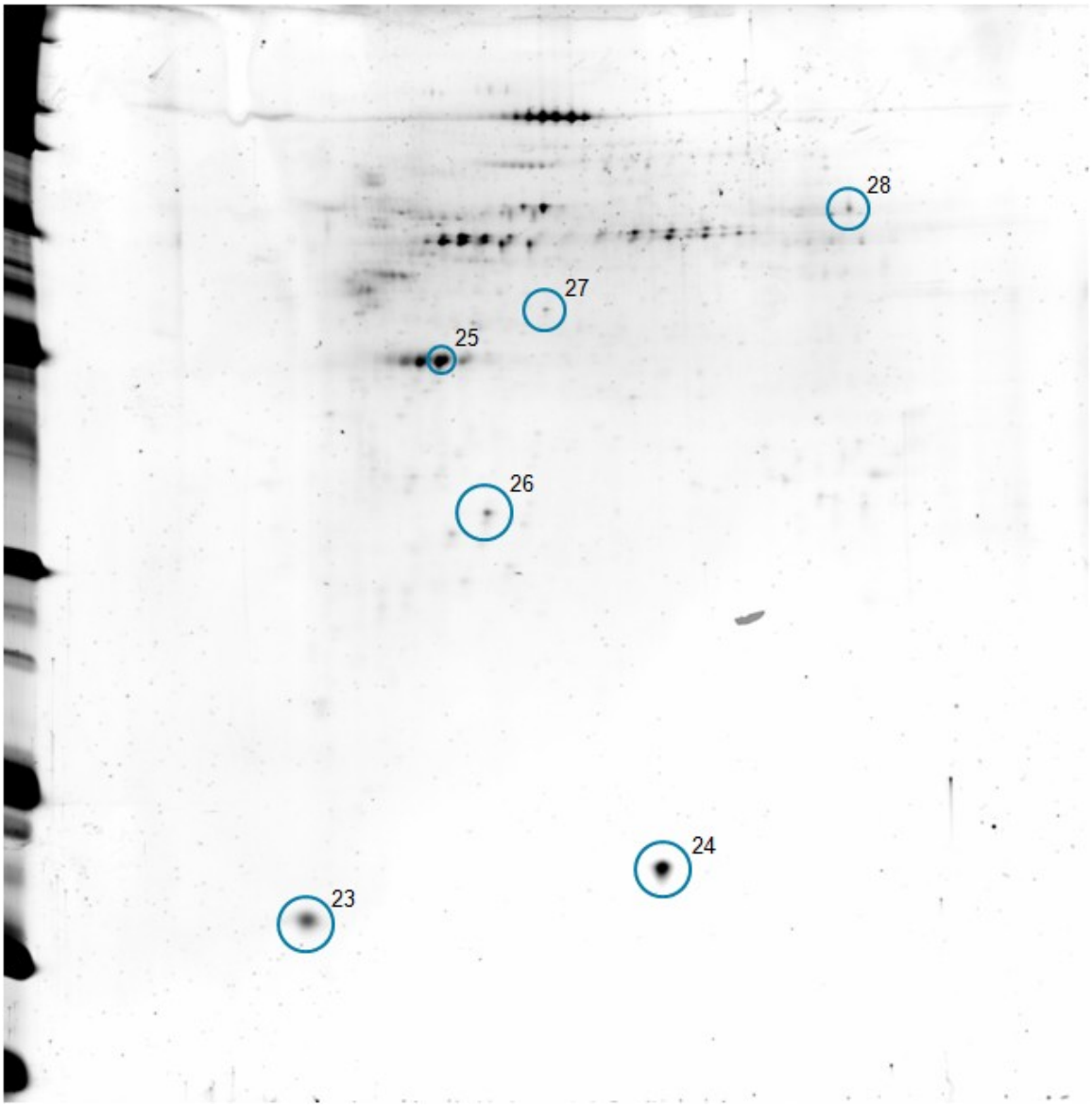

Figure 4.5. SYPRO Ruby stained secreted salivary proteome of RWA1.biotype of *Diuraphis noxia*. A total of 8 µg of protein was separated on a 3–10NL IPG strip in the first dimension, and on 12% SDS-PAGE in the second dimension. Spots were excised and subjected to nano-LC-MS-MS/MS.

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CHAPTER 5:

CONCLUSION AND FUTURE RESEARCH

This study ascribed function to candidate genes identified as differentially regulated during the wheat response to Russian wheat aphid infestation. *WRKY53,* a transcription factor, and *PAL*, a key enzyme in the phenylpropanoid pathway, were selected and gene silencing in aphid resistant germplasm was used to demonstrate that both genes are required for a successful resistance response. Since silencing of *WRKY53* also caused a decrease in *PAL* transcript abundance, *PAL* might be a downstream component of the *WRKY53* transcriptional network. An extensive set of yeast one- and two-hybrid assays were carried out to discover upstream components of the *WRKY53* signaling network, as well as proteins that might directly interact with the transcription factor. Many of these *trans*-acting genes are involved in signaling, phytoalexin synthesis, senescence and oxidative stress responses, which correlates with the described roles for orthologs of *WRKY53* in other plant species.

Since yeast hybrid screens are performed using the contrived environment of an engineered yeast cell, future work needs to be conducted to independently validate the interactions observed. DNA-binding interactions uncovered in yeast one-hybrid assays can be verified *in vivo* using chromatin immunoprecipitation (ChIP) (Park, 2009), which was recently successfully used to demonstrate the binding of AtWRKY33 to its own promoter (Mao *et al.*, 2011). Bimolecular fluorescence complementation (BiFC) (Walter *et al.*, 2004) is a useful approach toward validating protein–protein interactions

discovered in yeast two-hybrid assays, and has been used to confirm the interaction of the senescence-related AtWRKY53 with MEKK1 (Miao *et al.*, 2008).

Technical problems prevented the demonstration of binding of recombinant TaWRKY53 to W-box containing DNA probes derived from putative downstream target genes. Future research should focus on ascertaining the capacity of TaWRKY53 to bind to these promoters, helping to uncover more components of the TaWRKY53 activation network. It would also be interesting to investigate whether any of the other elements, such as the chitinase, peroxidase, DUF584 protein and glutathione *S*-transferase described in this study are differentially regulated when the expression of *TaWRKY53* is knocked down via virus-induced gene silencing (VIGS). Since WRKY transcription factors are targets for phosphorylation cascades, it might also be of interest to study how the different phosphorylation states of TaWRKY53 regulate its activity in the context of aphid defense.

The best possible technology available to any lab to date was used for the aphid saliva discovery proteomics, allowing for a really comprehensive characterization of the proteins secreted by two different Russian wheat aphid biotypes into artificial diet. All aphids clearly have salivary proteins in common. Salivary gland proteomes are complex, but the secreted proteomes detected using current technology and artificial diets are much less complex. The simplified artificial diets do not provide the same gustatory feedback to aphids, and it is therefore possible that secretomes collected from the diet do not accurately reflect the composition of saliva secreted *in planta*. However, a number of unusual proteins were indeed uncovered from secreted *D. noxia* saliva. It would be interesting to express these in plants to see if they are elicitors or effectors. Insects and plants do not interact with one another in isolation in a sterile environment. It is plausible that *D. noxia* inoculate bacteria or bacterial proteins into plants, which would induce an SA-mediated senescence response, mobilize chloroplast nitrogen back into the

phloem, and enrich their diet. SA and JA responses are antagonistic, which would cause the downregulation of JA/ET responses known to be effective against herbivores. This might explain why genes involved in both SA and JA signaling are differentially regulated during plant responses to aphids. Perhaps aphids use the inappropriate induction of SA responses to effectively distract the plant defense repertoire from launching a JA-based response more effective against insect herbivores, and also induce a modulated form of senescence conducive to aphid feeding. We now know from our studies of the WRKY53 transcriptional network that resistant plants are able to maintain regulatory control of the senescence response, preventing chlorosis from occurring. More extensive work needs to be conducted to ascertain not only that aphids are actively inoculating plants with microbes, but that this contributes a fitness benefit to phloem feeding insects. If new evidence can be found to support this mechanism, it would certainly precipitate a radical rethinking of current models of plant–insect interactions.

Although salivary proteins secreted into artificial diets have been extensively studied, an artificial diet remains a simplified approximation of the complex and dynamic composition of plant phloem. Aphids respond to sensory feedback from their food source, and may therefore alter the composition of their saliva based on the content of the diet. The technique also does not separate out the contributions of gel-like and E1 and E2 watery salivation periods to the final sample, although E2 saliva was likely not present in the samples collected in this study. Since overexpression of a salivary effector protein from *Myzus persicae* in *Nicotiana benthamiana* leads to SGT1-mediated chlorosis (Bos *et al.*, 2010), it might be worthwhile to investigate whether *D. noxia*-induced chlorosis also requires the expression of SGT1 in wheat, perhaps via VIGS. It might be interesting to clone and express the aphid proteins we have discovered, preferably using a baculovirus-assisted insect cell expression system which allows for appropriate post-translational modification (Kost *et al.*, 2005), and then inject these

expressed proteins into wheat to see if they act as effectors (causing chlorosis and leaf rolling) or elicitors (inducing resistance responses). Aphid proteins could also be cloned and constitutively expressed *in planta* to investigate their function, using rice as a model crop amenable to transformation.

A long-term goal of this study and research in cereal–aphid interactions in general would be to construct an extensive protein–protein interaction topology network composed of all interacting gene products from both plant and insect. Current and future technology will enable us to build a comprehensive picture of the dynamic and complex molecular interactions that allow aphids to subvert plants for their own survival, and plants in turn to resist the onslaught of aphid attack.

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CHAPTER 6:

SUPPLEMENTARY MATERIALS

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   1 ccctgctcct cccgtcgctc gatcgccATG TCCTCCTCCA CGGGGAGCTT GGACCACGCA
  61 GGGTTCACGT TCACGCCGCC GCCGTTCATC ACGTCCTTCA CCGAGCTTCT GTCGGGGTCC
 121 GGCGCCGGCG ACGCGGAGCG GTCGCCGAGG GGGTTCAACC GAGGGGGCCG GGCCGGGGCG
 181 CCCAAGTTCA AGTCCGCGCA GCCGCCCAGC CTGCCCATCT CGTCGCCCTT CTCCTGCTTC
 241 TCCGTCCCTG CAGGTCTCAG CCCTGCCGAG CTGCTCGACT CCCCCGTTCT CCTCAACTAC
 301 TCTCACATCT TGGCGTCTCC GACTACCGGT GCGATCCCTG CGCAGAGGTG CGACTGGCAG
 361 GCGAGCGCCG ATCTGAACAC CTTTCAGCAG GATGAGCTCG GCCTCTCTGG CTTCTCCTTT
 421 CACGCAGTCA AGTCCAACGC CACGGTCAAC GCTCAAGCAA ACCGCTTACC TTTATTCAAG
 481 GAGCAGCAGG AGCAACAACA AGAAGAAGTG GTTCAAGTGA GCAACAAGAG CAGCAGCAGC
 541 GGCAACAACA AGCAGGTTGA GGACGGATAC AATTGGAGGA AGTACGGGCA GAAGCAAGTT
 601 AAGGGGAGCG AGAACCCGCG GAGCTACTAC AAGTGCACCT ACAACAATTG CTCCATGAAG
 661 AAGAAAGTGG AGCGCTCTCT CGCCGACGGC CGCATCACGC AGATCGTCTA CAAGGGCGCA
 721 CATGACCACC CGAAGCCCCT CTCCACGCGC CGCAACTCCT CCGGCTGCGC GGCGGTCGTT
 781 GCGGAGGATC ATACCAACGG CTCGGAGCAC TCTGGCCCGA CGCCCGAGAA TTCATCCGTC
 841 ACTTTCGGAG ACGATGAGGC CGACAAGCCC GAGACCAAGC GCCGGAAGGA GCATGGTGAC
 901 AACGAGGGCA GTTCAGGCGG CACCGGCGGC TGCGGGAAGC CCGTGCGCGA GCCCAGGCTC
 961 GTGGTGCAGA CGCTGAGCGA TATAGACATA CTCGACGACG GCTTCCGGTG GAGGAAGTAC
1021 GGGCAGAAGG TTGTCAAGGG CAATCCCAAC CCCAGGAGCT ACTACAAGTG CACAACGGTG
1081 GGCTGCCCGG TGCGCAAGCA TGTGGAGCGG GCCTCGCACG ACAACCGCGC GGTGATCGCC
1141 ACCTACGAGG GTAAGCACAG CCACGACGTG CCGATCGGCC GGGGCCGCGC GCTGCCGGCG
1201 TCATCTTCCT CCGACAGCTC GGCCGTCATC TGGCCTGCCG CCGCCGTGCA AGCCCCGTGC
1261 ACCCTCGAGA TGCTCGCCGG ACACCCAGGC TACGCGGCCA AGGACGAGCC CCGGGACGAC
1321 ATGTTCGTCG AGTCGCTCCT CTGCTAGcta ggcaggctag gccgcggccc ttcgttcccc
1381 ctgtggcgtt tacatgtggg tccacg
```

Figure S1. Nucleotide sequence from a full-length cDNA clone of *TaWRKY53* isolated from hexaploid wheat (*Triticum aestivum* L.) cv. 'Gamtoos-R'. The initiation and stop codons are highlighted in bold; the binding sites for primers Ta.WRKY53_CDS_fwd and Ta.WRKY53_CDS_rvs are underlined.

```
   1 ccctgctcct cccgtcgctc gatcgccATG TCCTCCTCCA CGGGGAGCTT GGACCACGCA
  61 GGGTTCACGT TCACGCCGCC GCCGTTCATC ACGTCCTTCA CCGAGCTTCT GTCGGGGTCC
 121 GGCGCCGGCG ACGCGGAGCG GCCGCCGAGG GGGTTCAACC GAGGGGGCCG GGCCGGGGCG
 181 CCCAAGTTCA AGTCCGCGCA GCCGCCCAGC CTGCCCATCT CGTCGCCCTT CTCCTGCTTC
 241 TCCGTCCCTG CAGGTCTCAG CCCTGCCGAG CTGCTCGACT CCCCCGTTCT CCTCAACTAC
 301 TCTCACgtac gcctcctcca tctcgtgtgg ctgttgtgaa aaacttttt gctagtttcg
 361 tagtatgtgc atatgatccg tggtgctgat tttagctgcc gcctgcgttt ctgtcagATC
 421 TTGGCGTCTC CGACTACCGG TGCGATCCCT GCGCAGAGGT GCGACTGGCA GGCGAGCGCC
 481 GATCTGAACA CCTTTCAGCA GGATGAGCTC GGCCTCTCTG GCTTCTCCTT TCACGCAGTC
 541 AAGTCCAACG CCACGGTCAA CGCTCAAGCA AACTGCTTAC CTTTATTCAA GGtactccat
 601 aatagtacaa agataagata ttcagtatta ttgtcgaaac tactgtactt acataagatg
 661 cttcaacgtc tgaacttagt ttaactgtta cagcatgcac cgaggctgta tgctagtact
 721 aatttgttat gctttttat ctctctgaca atacagtcgt atctcacaaa gacgtacatg
 781 tttatgcagg AGCAGCAGGA GCAACAACAA GAAGAAGTGG TTCAAGTGAG CAACAAGAGC
 841 AGCAGCAGCA GCGGCAACAA CAAGCAGGTT GTGGACGGAT ACAATTGGAG GAAGTACGGG
 901 CAGAAGCAAG TTAAGGGGAG CGAGAACCCG CGGAGCTACT ACAAGTGCAC CTACAACAAT
 961 TGCTCCATGA AGAAGAAAGT GGAGCGCTCT CTCGCCGACG GCCGCATCAC GCAGATCGTC
1021 TACAAGGGCG CACATGACCA CCCGAAGCCC CTCTCCACGC GCCGCAACTC CTCCGGCTGC
1081 GCGGCGGTCG TTGCGGAGGA TCATACCAAC GGCTCGGAGC ACTCTGGCCC GACGCCCGAG
1141 AATTCATCCG TCACTTTCGG AGACGATGAG GCCGACAAGC CCGAGACCAA GCGCCGGtaa
1201 gtaattgatc attgcttgcc aaatattacc tttgtaaagt aatactcagt gatggcgcgt
1261 tccgctgcag tttgctttct catacgtgtt gttagattaa tttgaagatt gtgtgtttga
1321 ttggttgtca ggAAGGAGCA TGGTGACAAC GAGGGCAGTT CAGGCGGCAC CGGCGGCTGC
1381 GGGAAGCCCG TGCGCGAGCC CAGGCTCGTG GTGCAGACGC TGAGCGATAT AGACATACTC
1441 GACGACGGCT TCCGGTGGAG GAAGTACGGG CAGAAGGTTG TCAAGGGCAA TCCCAACCCC
1501 AGGtgagaat taccttcgat cagtagggaa ctcgtgatca gacttgtatc gtgacgacgt
1561 tgtcaatctg ttcgaccctg tgttcttact tgtatgatcg tgacgacgtt gcaggAGCTA
1621 CTACAAGTGC ACAACGGTGG GCTGCCCGGT GCGCAAGCAT GTGGAGCGGG CCTCGCACGA
1681 CAACCGCGCG GTGATCACCA CCTACGAGGG TAAGCACAGC CACGACGTGC CGATCGGCCG
1741 GGGCCGCGCG CTGCCGGCGT CATCTTCCTC CGACAGCTCG GCCGTCATCT GGCCTGCCGC
1801 CGCCGTGCAA GCCCCGTGCA CCCTCGAGAT GCTCGCCGGA CACCCAGGCT ACGCGGCCAA
1861 GGACGAGCCC CGGGACGACA TGTTCGTCGA GTCGCTCCTC TGCTAGctag gcaggctagg
1921 ccgcggccct tcgttccccc tgtggcgttt acatgtgggt ccacg
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Figure S2. Nucleotide sequence of a full-length genomic clone of *TaWRKY53* isolated from hexaploid wheat (*Triticum aestivum* L.) cv. 'Gamtoos-R'. Exons are in capitals; initiation and stop codons are highlighted in bold. The binding sites for primers Ta.WRKY53_CDS_fwd and Ta.WRKY53_CDS_rvs are underlined.

1 MSSSTGSLDHAGFTFTPPPFITSFTELLSGSGAGDAER**S**PRGFN

45 RGGRAGAPKFKSAQPPSLPIS**S**PFSCFSVPAGL**S**PAELLD**S**PVL

89 LNYSHILA**S**PTTGAIPAQRCDWQASADLNTFQQDELGLSGFSFH

133 AVKSNATVNAQANRLPLFKEQQEQQQEEVVQVSNKSSSSGNNKQ

177 VEDGYN**WRKYGQK**QVKGSENPRSYYKCTYNNCSMKKKVERSLAD

221 GRITQIVYKGAHDHPKPLSTRRNSSGCAAVVAEDHTNGSEHSGP

265 TPENSSVTFGDDEADKPETKRRKEHGDNEGSSGGTGGCGKPVRE

309 PRLVVQTLSDIDILDDGFR**WRKYGQK**VVKGNPNPRSYYKCTTVG

353 CPVRKHVERASHDNRAVIATYEGKHSHDVPIGRGRALPASSSSD

397 SSAVIWPAAAVQAPCTLEMLAGHPGYAAKDEPRDDMFVESLLC*

Figure S3. Translated peptide sequence from a full-length *TaWRKY53* cDNA clone isolated from hexaploid wheat (*Triticum aestivum* L.) cv. 'Gamtoos-R'. The protein consists of 439 amino acid residues. The five putative N-terminal phosphorylation sites are highlighted in bold, the two conserved WRKY domains are highlighted in bold, the zinc-finger motif is underlined and the nuclear localization signal is boxed.

Figure S4. ClustalW 2.0 multiple sequence alignment of the putative WRKY53 peptide sequence from 9 different wheat accessions.

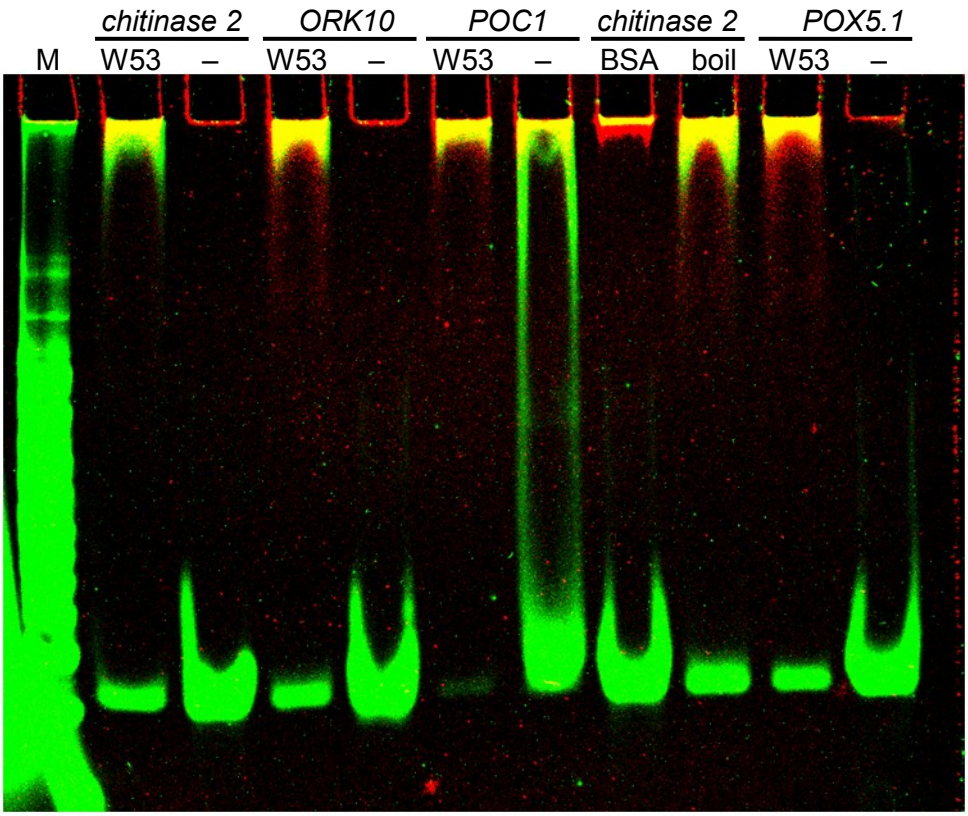

Figure S5. Dichromatic electrophoretic mobility shift assay. Nucleic acids are stained in green, proteins are stained in red.

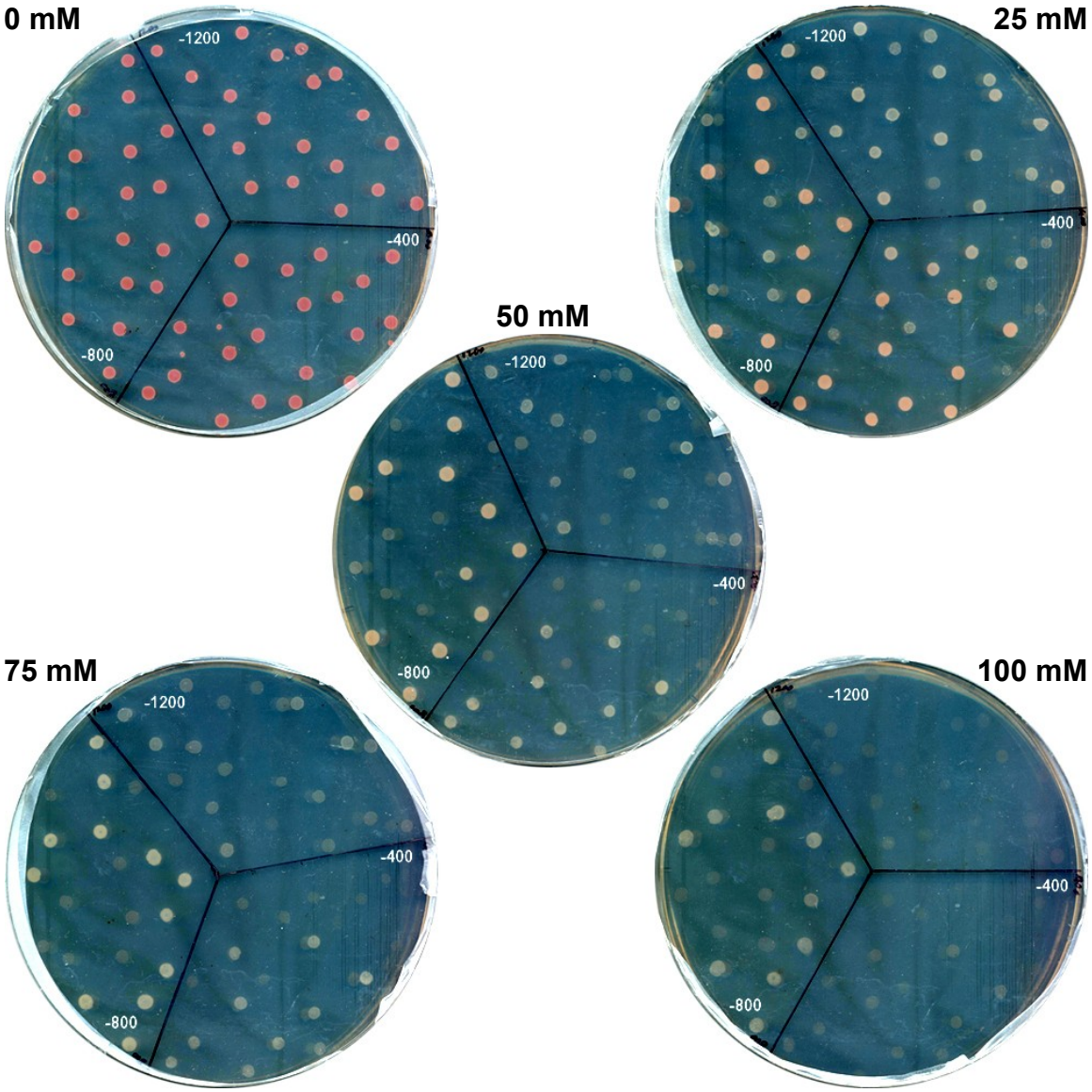

Figure S6. Yeast one-hybrid autoactivation assay. Colonies from each of the –400:P$_{W53}$, –800:P$_{W53}$ and –1200:P$_{W53}$ DNA bait strains were replica plated onto SD/–His/–Ura media supplemented with 0, 25, 50, 75 or 100 mM 3-amino–1,2,4-triazole (3-AT), a competitive inhibitor of the *HIS3* reporter gene product. Colonies exhibiting minimal growth at the lowest possible level of 3-AT were selected for yeast one-hybrid assays.

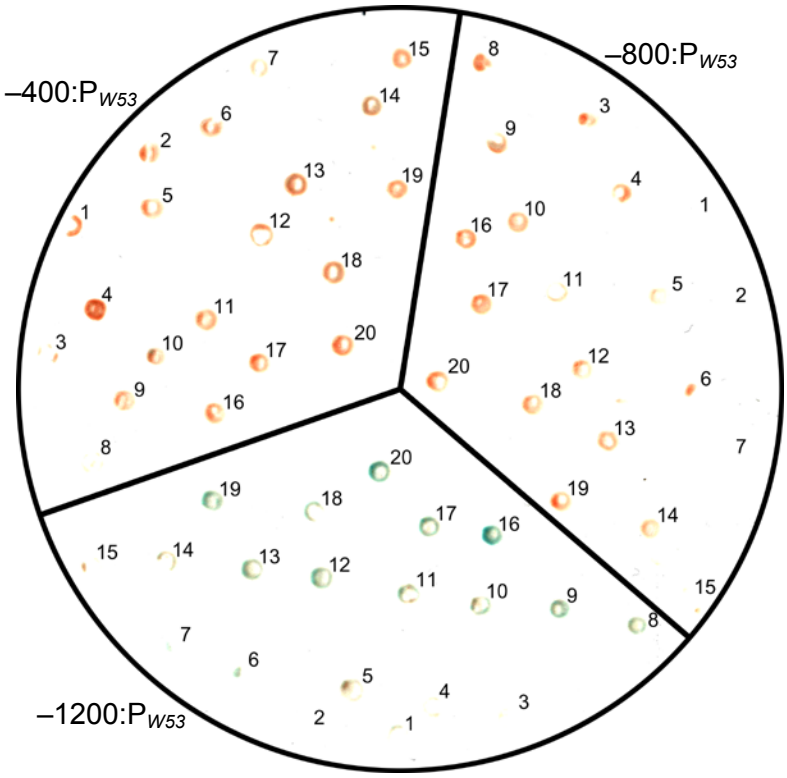

Figure S7. *β*-galactosidase assay indicating various levels of autoactivation of the *lacZ* reporter gene in 20 clones for each of the three yeast one-hybrid DNA bait strains –400:P$_{W53}$,–800:P$_{W53}$, and –1200:P$_{W53}$.

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AAGACGCGCACATCTGACTCACTTGGTGCACCTTGCCATGGGGAGCATGGACTGAGCAACAATTTTCAGAAAAAGAAAAATATTAAGCA
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TGTACACGACGGGAAAAAGACAAGGAACAAGCATAACGAATCATACGACCATTTCTTGATCGTGCCATTTTTGTTACCAGCAGCAACAT
AAGTTTTGCGCCAAGTGCAGAGTCGGCAATGACACTACAGGAAATTGACCGGTTTGCTTCCCTCTGTTTGACAGGTTCCTTAACGCCGG
TGCCTTCGGAACTGGCACGGACGGACACGTTCTGCCCGCCGAGGCGACACGCGCGGCTATACTCGTCCGCATCAACACCCTCCTGCAGG
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>PAL_GW_frag2.4
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TTTTACTCTAGAATTTGAATAAAATTCAACAGTACTAGTAGTATAATTTACTTTGTGCAGACCTCAAGTATTTATTCCCTAGTAGTATA
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>PAL_GW_frag3.2
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>PAL_GW_frag3.3
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CGGACACGTTCTGCCCGCCGAGGCAACACGCGCGGCTATGCTCGTCCGCATCAACACCCTCCTCCAGGGTTACTCGGGCATCCGCTTCG
AGATCCTCGAGGCCATCACCAAGCTGCTCAACGCCAATGTCAC

>PAL_GW_frag4.2
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CCGGTGCCTTCGGGACTGGCACGGACGGACACGTTCTGCCCGCCGAGGCGACACGCGCGGCTATGCTCGTCCGCATCAACACCCTCCTG
CAGGGTTACTCTGGCATCCGCTTCGAGATCCTCGAAGCCATCACCAAGCTGCTCAACGCCAATGTCAC

Figure S8. Nucleotide sequences from 5' genome walking clones obtained for the inducible *phenylalanine ammonia-lyase* gene (accession AY005474).

Table S1. Rice loci coregulated with *OsWRKY53*.

Locus ID	W-Boxes	Functional Annotation
Os01g61080	8	OsWRKY24
Os03g47280	6	VQ domain containing protein, putative, expressed
Os01g02300	5	receptor kinase ORK10, putative, expressed
Os01g27590	5	transposon protein, putative, Pong sub-class, expressed
Os03g58010	5	acetyltransferase
Os05g50180	5	OsCML14 - Calmodulin-related calcium sensor protein, expressed
Os01g03690	4	TKL_IRAK_DUF26-lg.1 - DUF26 kinases have homology to DUF26 containing loci, expressed
Os02g09960	4	Lyk8
Os03g37090	4	expressed protein
Os03g52410	4	expressed protein
Os04g46240	4	AP2 domain prot
Os05g41780	4	AP2 domain prot
Os05g46840	4	proline-rich protein, putative, expressed
Os06g13180	4	metalloendoproteinase 1 precursor, putative, expressed
Os07g48050	4	peroxidase precursor, putative, expressed
Os08g04370	4	plastocyanin-like domain containing protein, putative, expressed
Os11g36200	4	receptor-like protein kinase 2 precursor, putative, expressed
Os11g47600	4	glycosyl hydrolase (chitinase)
Os12g41110	4	OsCML5 - Calmodulin-related calcium sensor protein, expressed
Os01g06280	3	TKL_IRAK_CrRLK1L-1.4 - The CrRLK1L-1 subfamily has homology to the CrRLK1L homolog, expressed
Os01g46800	3	OsWRKY15 - Superfamily of TFs having WRKY and zinc finger domains, expressed
Os01g56240	3	OsSAUR2 - Auxin-responsive SAUR gene family member, expressed
Os01g67810	3	transposon protein, putative, unclassified, expressed
Os01g74250	3	TIGR01615 protein
Os02g02600	3	serine/threonine-protein kinase Cx32, chloroplast precursor, expressed

Os02g03410	3	CAMK_CAMK_like.12 - CAMK includes calcium/calmodulin depedent protein kinases, expressed
Os02g11859	3	expressed protein
Os02g13220	3	F-box family protein, putative, expressed
Os02g15810	3	HMG1/2
Os02g22160	3	DNA binding protein, putative, expressed
Os02g37330	3	heavy metal associated domain containing protein, expressed
Os02g45780	3	C3HC4 zinc finger
Os03g02514	3	hydrolase, alpha/beta fold family protein, putative, expressed
Os03g15770	3	tyrosine protein kinase domain containing protein, putative, expressed
Os05g01940	3	zinc finger, RING-type, putative, expressed
Os05g39930	3	spotted leaf 11, putative, expressed
Os06g04230	3	expressed protein
Os07g10970	3	leucine zipper protein-like, putative, expressed
Os07g39720	3	expressed protein
Os07g48010	3	peroxidase precursor, putative, expressed
Os09g37080	3	expressed protein
Os01g28790	2	PRAS-rich protein, putative, expressed
Os01g38980	2	calmodulin-binding protein, putative, expressed
Os02g33590	2	U-box domain-containing protein, putative, expressed
Os02g33680	2	U-box domain containing protein, expressed
Os02g50490	2	endoglucanase, putative, expressed
Os02g54600	2	STE_MEK_ste7_MAP2K.5 - STE kinases
Os03g53020	2	helix-loop-helix DNA-binding domain containing protein, expressed
Os03g58020	2	acetyltransferase, GNAT family, putative, expressed
Os04g03920	2	expressed protein
Os04g33390	2	prephenate dehydratase domain containing protein, expressed
Os04g34030	2	U-box domain-containing protein, putative
Os04g34050	2	VQ domain containing protein, putative, expressed

Os05g03620	2	TKL_IRAK_CR4L.4 - The CR4L subfamily has homology with Crinkly4, expressed
Os05g08830	2	expressed protein
Os05g45410	2	HSF-type DNA-binding domain containing protein, expressed
Os06g14450	2	exo70 exocyst complex subunit family protein, putative, expressed
Os06g44010	2	OsWRKY28
Os07g34940	2	aspartic proteinase nepenthesin-1 precursor, putative, expressed
Os07g35280	2	TKL_IRAK_DUF26-lc.1 - DUF26 kinases have homology to DUF26 containing loci, expressed
Os07g47990	2	peroxidase precursor, putative, expressed
Os07g48020	2	peroxidase precursor, putative, expressed
Os07g48030	2	peroxidase precursor, putative, expressed
Os07g48280	2	expressed protein
Os08g37660	2	plastocyanin-like domain containing protein, putative, expressed
Os08g42030	2	peroxidase precursor, putative, expressed
Os09g30490	2	EF hand family protein, expressed
Os11g11960	2	disease resistance protein RPM1, putative, expressed
Os12g36880	2	pathogenesis-related Bet v I family protein, putative, expressed (PR-10)
Os01g34450	1	expressed protein
Os01g50410	1	STE_MEKK_ste11_MAP3K.6 - STE kinases
Os01g57740	1	expressed protein
Os01g67820	1	exo70 exocyst complex subunit domain containing protein, expressed
Os02g08440	1	OsWRKY71
Os02g33600	1	VQ domain containing protein, putative
Os02g43820	1	AP2 domain containing protein, expressed
Os02g56370	1	OsWAK20 - OsWAK receptor-like protein kinase, expressed
Os02g56700	1	dehydrogenase, putative, expressed (cinnamoyl CoA reductase)
Os03g01740	1	expressed protein
Os03g04070	1	no apical meristem protein, putative, expressed

Os03g44380	1	9-cis-epoxycarotenoid dioxygenase 1, chloroplast precursor, putative, expressed
Os03g50280	1	GLTP domain containing protein, putative, expressed
Os03g50410	1	lipase family protein
Os03g55180	1	DUF1336 domain containing protein, expressed
Os03g55800	1	cytochrome P450, putative, expressed
Os04g34140	1	U-box protein CMPG1, putative, expressed
Os05g08860	1	expressed protein
Os05g46830	1	proline-rich protein, putative, expressed
Os06g09310	1	zinc finger, C3HC4 type domain containing protein, expressed
Os07g32940	1	hypothetical protein
Os07g48060	1	peroxidase precursor, putative, expressed
Os07g48770	1	serine hydrolase domain containing protein, expressed
Os08g40690	1	glycosyl hydrolase, putative, expressed (chitinase)
Os11g02369	1	LTPL7 - Protease inhibitor/seed storage/LTP family protein precursor, expressed (PR-14)
Os11g09010	1	lipase, putative, expressed
Os12g02310	1	LTPL11 - Protease inhibitor/seed storage/LTP family protein precursor, expressed (PR-14)