INVESTIGATING INSECT MOLECULAR RESPONSES TO TWO PLANT DEFENSE PROTEINS AND CHARACTERIZING A NOVEL INSECTICIDAL PROTEIN FROM *ARABIDOPSIS*

A Dissertation

by

YILIN LIU

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

December 2005

Major Subject: Entomology

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ABSTRACT

Investigating Insect Molecular Responses to Two Plant Defense Proteins and Characterizing a Novel Insecticidal Protein from *Arabidopsis*.

(December 2005)

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The molecular interaction between plants and insects is dynamic and multifaceted. We are interested in understanding the molecular mechanism that insects utilize to overcome plant defense proteins, as well as discovering novel plant insecticidal proteins. Three projects were developed. First, we evaluated the effects of soybean cysteine protease inhibitor (soyacystatin N, scN) on the growth and development in southern corn rootworm. Both subtractive suppressed hybridization (SSH) and cDNA microarray analyses were used to uncover the changes of gene expression profiles in southern corn rootworm under the scN challenge. The counterdefense-related genes were identified, suggesting that southern corn rootworm deployed several regulatory mechanisms to overcome the dietary scN. Second, to identify and confirm insecticidal properties of vegetative storage protein 2 in *Arabidopsis* (AtVSP2), the gene was cloned and expressed in *E.coli*. This protein showed acid phosphatase activity. Feeding assay indicated that AtVSP increased the mortality and delayed the development of two coleopteran and one dipteran insects. Third, to identify the molecular mechanism of this

novel insecticidal protein, *P* element mutagenesis was utilized to generate AtVSP resistant mutants (VRs). Two balanced VR mutants and their revertants were generated, and can be used to further characterize the genetic loci of *P* element inserted in the mutants.

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CHAPTER I

INTRODUCTION

1.1 Co-evolution between plants and insects

Plants and insects have coexisted and coevolved for as long as 350 million years. Although some relationships developed between the two phyla, such as pollination, are mutually beneficial, the most common interactions involve insect predation and the defense of corresponding plants against such herbivory (Gatehouse, 2002). From a plant's perspective, success in this interaction is measured by the ability to defend itself from devastation by insect feeding. From an insect's perspective, success is measured by the ability to protect itself from a variety of toxic plant defense compounds, thereby allowing it to utilize plants as a primary food source. These interactions are multifaceted and dynamic. Many classes of insect repellents and toxic substances, including a series of allelochemicals and proteins, are synthesized in plants. The biosynthetic pathways leading to these substances are continually evolving to generate new toxic compounds. From the insect side, a variety of adaptation mechanisms, including enzymatic detoxification systems, physiological tolerance and behavioral avoidance, protect insects from the hazard of plant toxic compounds. These mechanisms continue to evolve as insects attempt to colonize new plant species (Schuler, 1996). As a consequence, both plant defense and insect adaptation involve a metabolic cost. In a natural system, most

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This dissertation follows the style and format of Insect Biochemistry and Molecular Biology.

plant–insect interactions reach a ‘stand-off’ where both hosts and herbivores survive but develop suboptimally (Gatehouse, 2002). The plant-insect interactions have been described in detail in ecological and entomological literature. Here I briefly review the current understanding of this relationship, with a particular focus on its molecular and genetic basis.

1.2. The complexities of plant defense responses

1.2.1 Diverse defense responses in plants

Plant responses to herbivorous pests were extensively studied and generally categorized as direct defense, indirect defense and tolerance (Kessler and Baldwin, 2002). Direct defenses involve physical barriers, such as thorns and silica, as well as syntheses of toxic chemicals (alkaloids, terpenoids, phenolics), repellents, antinutritients, and antidigestive compounds. Indirect defense refers to the mechanisms utilized by plant to attract predators and parasitoids of herbivores and to increase foraging success of the natural enemies, thereby facilitating top-down control of herbivore populations (Kanban and Baldwin, 1997). A typical example of indirect defenses is volatile organic compounds (VOCs) released by herbivore-attacked plants (Dicke and van Loon, 2000). Tolerance allows a plant to sustain tissue losses with little or no decrease in fitness relative to that in an undamaged state (Stowe et al., 2000). Although plant species

clearly vary in their tolerance, the mechanisms underlying such variation are not understood (Kessler and Baldwin, 2002).

The advent of new tools for genomic studies, such as available genome sequences of model organisms, databases of expressed sequence tags (ESTs), differential display and microarrays, has allowed a more thorough investigation of the changes in gene expression profiles occurring in plant defense responses. Microarray-based study monitored differential regulation of 150 defense-related *Arabidopsis* genes by insect attack (Reymond et al., 2000). Approximately 500 mRNAs constitute the insect-responsive transcriptome in tobacco (Hermsmeier et al., 2001). Eighty sorghum genes were found to respond to greenbug attack (Zhu-Salzman et al., 2004). A recent study identified 114 genes in *Arabidopsis* in response to *Pieris rapae*, of which 111 were induced and only three were repressed (Reymond et al., 2004). Generally, genes involved are those functioning in defense, secondary chemical metabolism, signal transduction, detoxification, abiotic stress, cell survival, photosynthesis, and genes of unknown function. The complexity of the plant defensive responses is also reflected by changes in gene expression that are not directly involved in insect resistance and general stress response. Photosynthetic genes, for instance, were down regulated by the herbivore attacks. Such response enables plants to allocate more resources to produce defense proteins (Hermameier et al., 2001; Zhu-Salzman et al., 2004).

1.2.2 Multiple signal pathways regulate plant defense response

Recognition of the invading pest insect relies on several factors. Mechanical damaging as well as herbivore's elicitors, in combination with abiotic stresses, differentially activate a bewildering array of genes and transduction cascades (Walling, 2000). The signaling pathways leading to the production of insecticidal proteins involves several major molecules: systemin, jasmonic acid (JA), salicylic acid (SA) and ethylene (Ryan, 2000; Hui et al., 2003).

Systemin, the first signal in the wound response (Ryan and Pearce, 1998), is derived from proteolytic cleavage of a precursor polypeptide, prosystemin. The 18-amino-acid peptide molecule interacts with a receptor present on the surface of plant cells (Meindl et al., 1998). The signal transduction mediated by the systemin-receptor results in activation of phosphorylipase A2, via a MAP kinase, which causes the release of linolenic acid from membrane lipids. Linolenic acid acts as a precursor for the synthesis of jasmonic acid (JA).

JA is an oxylipid signaling molecule involved in stress and developmental responses in plants. Numerous reports have shown that the JA is a key regulator in protecting plants from insect attack in both the laboratory and the natural environment (Orozco-Cardenas et al., 1993; Howe et al., 1996; McConn et al., 1997; Stintzi et al., 2001). JA controls activation of downstream defense genes (Reymond et al., 2000; Schenk et al., 2000), participates in physical defense production (Heil et al., 2001), and

controls the production of volatiles that participate in indirect defense processes (Thaler et al., 2002; Schmelz et al., 2003; Van Poecke and Dicke, 2004).

Although JA is the central plant signal molecule for wound and herbivorous attack, it has become apparent that more signal molecules are associated with such responses. The recent reports illustrated the importance of the jasmonate, salicylate, and ethylene pathways during interaction between *Arabidopsis* and the generalist Egyptian cotton leafworm, *Spodoptera littoralis* (Stotz et al., 2000, 2002). Reymond and Farmer (1998) proposed a "tunable dial" model for the regulation of defensive gene expression, based on the crosstalk of these three signal pathways. According to this model, a plant tailors its defensive responses to a specific attacker by eliciting these signal molecules to different degrees. Synergistic as well as antagonistic effects among these hormonal regulators have been reported. The synergistic effects of JA and ethylene on the expression of defensive genes have been described in *Arabidopsis* (Penninckx et al., 1998). Meanwhile, it is also reported that ethylene antagonizes the JA-induced defense genes in *Griffonia simplicifolia* and *Arabidopsis* (Zhu-Salzman et al., 1998; Rojo et al., 1999). JA and ethylene are often antagonized by SA (Dong, 1998; Reymond and Farmer, 1998; Pieterse and van Loon, 1999). Further, other combinations have also been recorded, such as SA being synergized by JA and ethylene (Xu et al., 1994; Lawton et al., 1994; Reymond and Farmer, 1998; Pieterse and van Loon, 1999).

1.2.3. Plants use insect oral factors as cues for defense responses

The herbivore elicitor (insect saliva) is another important component to determine the plant defensive response. The saliva of some chewing insects often contain fatty acylated amino acids (Alborn et al., 1997; Halitschke et al., 2001) and/or enzymes, such as β-glucosidase (Mattiacci et al., 1995; Musser et al., 2002) that can powerfully influence gene expression in host via the jasmonate pathway. When salivary components are applied to mechanically wounded tissues, they either induce or repress the level of some transcripts (Halitschke et al., 2001, 2003; Schittko et al., 2001). For example, *Manduca sexta* is "recognized" by the plant as evidenced by a JA burst far greater than that produced by mechanical wounding (McCloud and Baldwin, 1997). Moreover, differences in the biochemical composition of insect saliva between species have been reported (Alborn et al., 2003), potentially leading to differential host responses to particular herbivore species (Korth and Dixon, 1997; Dicke, 1999).

Furthermore, there is a clear difference in defense responses between chewing caterpillars and phloem-sap-sucking whiteflies or aphids. These homopteran insect pests have a feeding habit that minimizes tissue damage, and thus are able to avoid much of the wounding response. Aphid feeding induces the expression of pathogen-, SA-, as well as wound- and JA-regulated genes (Walling, 2000). Evidence suggests that a particular combination of pathways is often highly specific to the damaging insect.

1.2.4 Insecticidal plant proteins

As a result of crosstalk, plants acquire various defense compounds to combat the herbivorous insect. Various proteins are integral parts of these substances: proteolytic enzymes, chitinase, polyphenol oxidase, protease inhibitors, α-amylase inhibitors, as well as lectins (Ryan, 1990; Peumans and Van Damme, 1995a; Fritig et al., 1998; Garcia-Olmedo et al., 1998). The proteolytic enzymes can be involved in plant defense reactions, such as enzyme turn over. The enzymes can also directly modify proteins and enzymes in the insect body (Mosolov et al., 2001). One example is a 33-kDa cysteine protease from resistant maize that is able to damage the peritrophic matrix in larvae of (*Spodoptera frugiperda*), leading to insect growth reduction (Pechan et al., 2002). The chitinases are presumed to target chitin structures such as the peritrophic membrane, a crucial sieve protecting the delicate midgut cells (Ding, 1998). Polyphenol oxidases generate toxic compounds by conjugating phenolics to proteins (Felton et al., 1992). Protease inhibitors and α-amylase inhibitors deprive the insects of nutrients by interfering with their digestive enzymes (Hilder et al., 1987; Huesing et al., 1991). Lectins constitute another large family of defensive proteins, whose toxicity is associated with their ability to bind glycosylated proteins in the insect midgut (Chrispeels and Raikhel, 1991; Harper et al., 1995).

1.3. Insects employ several strategies to adapt to plant defense compounds

As a consequence of co-evolution, herbivorous insects have adapted to plant defenses they normally encounter (Gatehouse, 2002; Glendinning, 2002), thereby developing a series of counter-defense strategies or mechanisms with behavioral, physiological, biochemical, or genetic aspects. One of the most common mechanisms that insects utilize is to inactivate the toxic plant compounds. For example, plants produce insect molting hormones, phytoecdysteroids (PEs), which either function as feeding deterrents or disrupt insect development (Kubo et al., 1983). However, some insects have adapted to PE by evolving efficient detoxification mechanisms via an ecdysteroid-22-O-acyltransferase and thus are unaffected by these dietary molting hormones (Zhang and Kubo, 1992, 1993). Cytochrome P450 monoxygenases and glutathione S-transferases are two main enzymes used by insects to detoxify toxic plant secondary metabolites (Yu, 1996; Feyereisen, 1999). These enzymes can breakdown or convert the toxic plant compounds into non-toxic products that can be excreted. It is reported that xanthotoxin induces P450 expression in corn earworm (*Helicoverpa zea)*, which helps the insect adapt to the dietary toxin (Li et al., 2004).

In addition to detoxifying plant defense compounds, many insects evade potentially toxic chemicals in plants in a proactive manner. In Brassicaceae, the glucosinolate–myrosinase system, also referred to as "the mustard oil bomb" is a sophisticated two-component system to ward off insect attack. Intact glucosinolates have limited biological activity. Their potency arises when plant tissue is damaged, and

glucosinolates come into contact with plant myrosinase. Myrosinase removes the β - glucose moiety from glucosinolates, leading to the formation of a variety of toxic products. However, this "mustard oil bomb" is disarmed by a crucifer specialist insect, the diamondback moth (DBM), *Plutella xylostella*. The glucosinolate sulfatase in DBM desulfates glucosinolates, rendering them invisible to plant myrosinase. Therefore, DBM can prevent the formation of toxic glucosinolate products (Ratzka et al., 2002). Furthermore, insects can perceive plant defense signal molecules, and synthesize the detoxifying enzymes while plants are producing defense molecules. For example, corn earworm use JA and SA to activate four of cytochrome P450 genes that are associated with detoxification either before or concomitantly with the biosynthesis of allelochemicals (Li et al., 2002).

Some insect species can even utilize plant toxins to their own advantage to repel their predators. *Estigmene acrea* is highly adapted to obtain alkaloids from any pyrrolizidine alkaloid (PA) containing plants. Four specific features demonstrate the ability of this species to adapt to PA: (i) broad and sensitive gustatory recognition of plant PAs; (ii) ability to convert and maintain the toxic PAs in a non-toxic state; (iii) transfer of the alkaloids ingested by larvae to the adult stage, and at oviposition these alkaloids are transmitted to the eggs (Dussourd et al., 1988); and (iv) transformation of the various plant acquired PA into the male courtship pheromone, hydroxydanaidal (Hartmann et al., 2004, 2005). Therefore, this herbivorous insect is unpalatable to predators and avoided by parasitoids because they possess poisonous defense compounds in their bodies.

1.4. Objectives of the work

It is estimated that the world population will reach 10 billion over the next four decades, which requires sustainable and efficient agriculture to provide substantial food and other produces (Ferry et al., 2004). However, losses of agricultural production due to pests and diseases have been estimated at 40% worldwide. Insects cause yield losses not only by herbivory, but by diseases they vector. At present, plant protection relies predominantly on the use of agrochemicals. Issues on human health and the environment necessitate exploration of alternatives (Boulter, 1993). Genetic control or the use of transgenic crops expressing insecticidal genes could make a significant contribution (Estruch et al., 1997). Although most current insecticidal plants have been based on *Bt* δ-endotoxins, many research projects are aimed at discovering non-*Bt* proteins to control insect pests. A number of these insecticidal proteins, such as polyphenol oxidases, protease inhibitors, lectins and α-amylase inhibitors, interfere with the nutritional needs of insects. These natural defense proteins, induced in certain plant tissues by herbivory or wounding, are well-established insecticidal proteins (Ryan, 1990; Koiwa et al., 1998; Franco et al., 2002). These genes are promising alternatives to the use of *Bt* insecticidal genes. However, the utilization of these plant defense proteins has some limitations. Protease inhibitors, chitinases and lectins show insecticidal properties at mg/ml (hundreds or thousands ppm) concentration (Boulter, 1993; Shah et al., 1995; Kanost and Jiang, 1996). Proteinase inhibitors and α-amylase inhibitors bind to the insect digestive enzymes and its binding does not directly elicit cell death. Thus a large

amount of proteins and a long-term, chronic exposure is needed to manifest any insecticidal property, which may give insects opportunities to develop adaptative mechanisms.

A key challenge is to identify novel genes that will produce insecticidal products with suitable characteristics for use in transgenic crops. Meanwhile, a great effort to understand insect counterdefense mechanisms is also likely to provide many benefits.

In this dissertation, two jasmonate-induced proteins (JIPs) were studied. The first is a proteinaceous protease inhibitor that is a well-characterized plant defense protein. The possible role of protease inhibitors (PIs) in plant protection was envisaged as early as 1947 when Mickel and Standish observed that the larvae of certain insects were unable to develop normally on soybean products (Haq et al., 2004). PIs are present in multiple forms in numerous tissues of animals and plants as well as in microorganisms. PIs have received ample attention because their small size, abundance, and stability make them easy to work with (Ryan, 1990). They are usually highly specific for a particular class of digestive enzymes. Soyacystatin N (scN) is a soybean cysteine protease inhibitor induced by wounding or by methyl jasmonate treatment (Botella et al., 1996; Zhao et al., 1996). While the insecticidal activity of scN has been observed (Koiwa et al., 1998) an adaptive feeding behavior of a coleopteran insect has also been reported (Zhu-Salzman et al., 2003). Studying the insect molecular responses to this protein could further our understanding of the insect adaptation mechanism.

The other JIP is a vegetative storage protein (VSP), functioning as a nitrogen reservoir during plant development in soybean (Mason and Mullet, 1990) but not

previously recognized as a defense compound. The VSP homologue in *Arabidopsis* is up regulated by JA treatment, wound and herbivore attacks, indicating the potential anti-insect function. AtVSP was expressed and characterized *in vitro* and its insecticidal activity was verified by this study. To dissect the insecticidal mechanism of AtVSP, *Drosophila P*-element mutagenesis can be a good method to locate the target sites.

CHAPTER II

TRANSCRIPTIONAL REGULATION IN SOUTHERN CORN ROOTWORM LARVAE CHALLENGED BY SOYACYSTATIN N*

2.1. Introduction

Southern corn rootworm (*Diabrotica undecimpunctata howardi* Barber) is one of the most damaging pests in the United States to major economic crops including corn (*Zea mays*) and peanut (*Arachis hypogaea*). The larvae bore into germinating seeds and also feed on the roots of many plant species causing severe damage, and the adult beetles are general feeders infesting more than 200 plant species and acting as vectors of several economically important viruses (Fabrick et al., 2002). Costs associated with crop loss and insecticide application to control rootworm damage to corn roots and corn silks are over one billion dollars annually (Metcalf, 1986; Fuller et al., 1997). Increased deregistration of soil insecticides, due to their environmental and human health consequences, has reached the point where rescue applications to control rootworm outbreaks are no longer possible for peanuts in the southern region. Genetic engineering of crop plants offers an environmentally friendly alternative for sustainable insect pest control.

Like other coleopterans, corn rootworms use cysteine proteases, a group of

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proteases that possess a catalytic triad composed of a cysteine, a histidine and an aspartate, as their major digestive enzymes (Gillikin et al., 1992; Edmonds et al., 1996; Koiwa et al., 2000; Fabrick et al., 2002; Bown et al., 2004). Cysteine protease activity accounts for most digestive activity in the midgut of southern corn rootworm larvae. Inhibition of this enzymatic activity resulted in growth retardation and increased mortality (Orr et al., 1994; Fabrick et al., 2002). With the plant transformation techniques available today, it is possible to engineer plants with novel insect resistance genes from many exogenous sources. Naturally-occurring plant defense genes like those encoding protease inhibitors have also received serious consideration as candidates for transgenic pest control. The defensive function of protease inhibitors is attributed to their ability to suppress insect protein digestive enzymes, which leads to a reduction in amino acid assimilation by insects. The soybean cysteine protease inhibitor, soyacystatin N (scN), suppresses digestive enzymatic activity of cowpea bruchid (*Callosobruchus maculatus*), western corn rootworm (*Diabrotica virgifera virgifera*), and Colorado potato beetle (*Leptinotarsa decemlineata*), as well as the growth and development of these pests (Zhao et al., 1996; Koiwa et al., 1998, 2000; Zhu-Salzman et al., 2003). These biochemical and insect feeding assays suggested that scN could be an additional candidate protein potentially useful in transgenic approaches for rootworm control.

However, insect adaptation to engineered plant resistance has become a major concern in using plant protease inhibitors for management of pest insects. Numerous crop plants have been transformed with protease inhibitors, but control has been

relatively limited or at best only transient. Studies have revealed that insects employ several strategies to avoid the effects of inhibitors. Some insects adapt to protease inhibitors by overproduction of existing digestive proteases (De Leo et al., 1998), while others selectively induce inhibitor-insensitive proteases (Bolter and Jongsma, 1995; Jongsma et al., 1995; Bown et al., 1997; Cloutier et al., 2000; Mazumdar-Leighton and Broadway, 2001; Zhu-Salzman et al., 2003). Furthermore, plant protease inhibitors may themselves be inactivated by direct proteolytic fragmentation (Michaud et al., 1995; Giri et al., 1998; Zhu-Salzman et al., 2003). When southern corn rootworm larvae fed on a diet containing potato multicystatin, high mortality was observed in younger insects. The toxic effect decreased when older insects were used in feeding experiments (Orr et al., 1994), suggesting that insect resistance to plant defense protease inhibitor could be developmentally regulated. Determination of the transcriptional regulation of inhibitor-responsive genes is thus a necessity in understanding insect adaptation to plant defense, as well as in employing insect resistance genes in transgenic plants.

In this paper, we evaluated the impact of scN on southern corn rootworm larval growth and development. We then used a combination of subtractive hybridization and cDNA microarray analysis to identify scN-regulated genes. We found that scN increased the mortality of southern corn rootworm in a dose-dependent manner. Growth retardation was more evident earlier in insect development. In response to scN in the diet, the larvae increased the expression of cysteine and aspartic proteases as well as peritrophin, and decreased expression of metabolic proteins. We determined that expression of these genes is also developmentally regulated.

2.2 Materials and methods

2.2.1. Purification of scN

Bacterially expressed recombinant scN was obtained following Koiwa et al. (2000). This method produces extremely pure, biologically active recombinant proteins, free of endotoxin that could possibly impact insect growth and development (Koiwa et al., 1998; Zhu-Salzman et al., 1998). Briefly, cells harbouring the scN expression cassette were grown in LB broth until OD600 reached 0.5 to 1.0, when isopropyl-D-thiogalactoside was added to the cell culture to a final concentration of 1.0 mM. Following an overnight incubation at 25°C, cells were harvested by centrifugation and disrupted by French press at 16,000 psi (Spectronic Instruments, Rochester, NY). Cell debris was removed by centrifugation, and the recombinant protein was purified by Ni2+-chelate sepharose affinity chromatography (Amercham Pharmacia Biotech). Purified scN was dialyzed exhaustively against distilled water and lyophilized.

2.2.2. Southern corn rootworm feeding assays

scN was incorporated into the insect diet to analyze its effect on mortality and development of southern corn rootworm larvae. Nondiapausing southern corn rootworm egg masses were purchased from French Agricultural Research Inc (French Agricultural Research). Diet for the rootworms was purchased and prepared as instructed by the

manufacturer (Bio-serv). Briefly, autoclaved water and agar were cooled to 55°C and kept in the water bath. Diet mix was then incorporated, mixed and dispensed as aliquots in titer-well plates. This was followed by immediate addition and mixing of a series of scN solutions of various concentrations, or distilled water for the control diet. Evenly mixed diet was allowed to solidify. To decrease chance contamination, all diet preparation and handling steps were done in a laminar flow hood. In addition, tetracycline (12.5 μg/mL) and carbenicillin (50 μg/mL) were included in all diets to prevent bacterial contamination. A total of 40 neonate larvae, in four replicates (10 larvae per replicate), were used for each scN concentration. Insects were reared in total darkness at 28°C, and transferred daily to new plates with fresh diets. Larval mortality as well as body weight of surviving insects were recorded and plotted using KALEIDA-GRAPH (Abelbeck software).

Survival data were recorded as percentage and were $\log_{10}$ transformed prior to further analysis (Sokal and Rohlf, 1995). The regression analysis procedure in SAS 2000 (SAS Institute Inc.) was used to calculate regression equations and R-square values for each treatment with larval developmental time as the dependent variable. User defined overall between-treatment linear contrasts were performed using PROC GLM (SAS 2000). Weight data were subjected to tests for the assumptions of normality. Where necessary, appropriate transformations were conducted to normalize distributions (Sokal and Rohlf, 1995). One-way analysis of variance (ANOVA) and Bonferroni multiple means comparisons tests were performed to analyze treatment effects (Sokal and Rohlf, 1995).

2.2.3. Construction of the subtractive cDNA library

Second instar larvae reared on scN diet at the concentrations of 0.1% and 0.25% as well as on scN-free diet, respectively, were collected for use in subtractive hybridization. Doses of scN were selected based on the bioassay results. Selected doses significantly impacted insect mortality and growth, yet allowed collection of sufficient materials to perform further experiments. Larvae were homogenized for mRNA extraction using a QuickPrep Micro mRNA Purification kit (Amersham Pharmacia Biotech). The PCR-Select cDNA Subtraction kit (Clontech) was used to obtain cDNAs corresponding to genes differentially regulated by dietary scN. Both forward and reverse subtractions were performed; the forward subtraction used tester cDNA obtained from mRNA of scN-fed rootworm larvae and driver cDNA from control larvae, and vice versa in the reverse subtraction. Subtracted cDNAs were subjected to two rounds of PCR amplification to normalize cDNA populations. The PCR products were ligated into pCRII-TOPO® vector (Invitrogen), and transformed into *E. coli*, DH10B™ cells (Invitrogen). A total of 1,920 bacterial clones were inoculated, amplified and maintained in freezer medium (50mM potassium phosphate, 2.0 mM sodium citrate, 0.8 mM $MgSO_4$, 7.0 mM $(NH_4)_2SO_4$, pH 7.5) with carbenicillin (50 μg/mL) in 96-well microplates.

2.2.4. DNA microarrays

cDNA inserts of the subtracted collections were PCR-amplified in 150 µL reactions in microplate format, using M13 forward and M13 reverse universal primers. PCR products confirmed by gel electrophoresis were precipitated by addition of 15 µL of 3 M NaOAc, pH 5.2, and 150 µL isopropanol per reaction at –20°C overnight. Microplates were centrifuged at 3,200 rpm for 2 hr at 4°C. Pellets were washed with 70% EtOH, vacuum dried, and resuspended in 35 µL spotting solution (2x SSC, 0.1% sarkosyl) each. DNA clones were printed onto polyL lysine-coated glass slides (CEL Associates, Houston, TX) using an Affymetrix 417 arrayer. Following printing, DNA was UV crosslinked at 550 mJ, and slides were further processed by blocking in 0.2% SDS for 10 min at 25^{o}C, followed by DNA denaturation in boiling H_2O for 2 min, and treatment with –20^{o}C ethanol (95%) for 2 min.

Microarray probes were synthesized from equal amounts of total RNA extracted from scN-fed and control 2nd instar larvae, respectively, using the 3DNA expression array system (Genisphere, Hatfield, PA). Primers containing oligo dT and a proprietary "capture sequence" for Cy3 or Cy5, respectively, were used with reverse transcriptase to synthesize cDNA probes. These probes (derived from 5 µg input RNA per channel for each microarray) were mixed with an equal volume of hybridization buffer (Express-hyb, Clontech), added to the microarray and covered with a cover slip, and the slides were sealed in aluminum hybridization chambers (Monterrey Industries, Monterrey, CA) for hybridization at 65°C overnight. After washing (2x SSC/0.1% SDS for 10min at 65°C,

followed by 10 min in 2x SSC and then 10 min in 0.2x SSC, both at 25^{o}C with shaking), a second hybridization was conducted to incorporate Cy3 or Cy5 fluor, respectively, which were coupled to an oligonucleotide complementary to the "capture sequence". Thus, cDNA probes bound to the microarray were fluor-labeled by hybridization.

Slides were scanned with a Packard Scanarray 5000 4-laser confocal scanner (Packard BioChip Technologies) using the Scanarray program. Scanning parameters were adjusted to obtain balanced signals on the two channels using the line-scan function. Image analysis was done with the Quantarray Program (Packard BioScience). Data from both channels were background subtracted and normalized by a Lowess algorithm using the GeneSpring 6.2 program (Silicon Genetics). Means and standard deviations of normalized and background-subtracted fold-change values from replicate experiments were derived in Microsoft Excel, and are presented in Table 2. For each given cDNA spot, gene expression was considered to be changed by the treatment if the spot had an average fold change ratio of >1.8 or <0.56 over the two treatments, and gave a signal intensity of >5000 in at least one of the two channels (control or treatment) in both replicates. When using 1.8-fold induction and suppression ratio cutoffs and a signal intensity cutoff of 5000, less than 0.2% of the whole array are expected to be miscategorized. This was established in our laboratory, using both insect and plant materials, by a series of self vs self microarray experiments in which Cy3- and Cy5-labeled probes made from the same RNA were co-hybridized to the DNA arrays (Moon et al., 2004; Zhu-Salzman et al., 2004). Further, a series of preliminary dye-swap experiments demonstrated that the labeling system used resulted in negligible dye bias,

as has been reported elsewhere (Yu et al., 2002). We therefore adopted the convention of using Cy5 fluor to label the control probe in all experiments.

2.2.5. DNA sequencing analyses

Differentially expressed cDNAs were subjected to dideoxy terminator cycle sequencing using the ABI BigDye sequencing kit (PE Biosystems), and analysed on an ABI Prism 3100 DNA sequencer. Sequencher^{TM} software (Gene Codes Corporation) was used to trim the vector sequence from raw sequence data, and to assemble contigs. cDNA identities were determined by sequence comparison to the GenBank database using BLASTX. For those sequences that did not result in any hits using BLASTX, BLASTN and tBLASTX searches were employed.

2.2.6. Northern blot analysis

Seven µg of total RNA from various developmental stages of control and scN-fed southern corn rootworm larvae were separated on 1.2% agarose formaldehyde gels, transferred to Hybond-N nylon membrane and hybridized with ^{32}P-labeled cDNA probes. Blots were washed (4x SSC/0.1% SDS, 2x SSC/0.1% SDS, 1x SSC/0.1% SDS, 0.5x SSC/0.1%SDS, 0.1% SSC/0.1% SDS at 65^{o}C), and exposed to X-ray film. The signals were detected by PhosphorImager and quantified by using ImageQuant 5.0 software (Molecular Dynamics, Inc.).

2.3. Results and discussion

2.3.1. scN negatively impacts growth and survival of southern corn rootworm

To assess the effect of scN on mortality and development of the rootworm larvae, the inhibitor was incorporated into the artificial diet at a series of concentrations and fed to neonate larvae. Linear regression R-square values and probabilities using log transformed survival percent data indicated good linear fits, justifying further general linear model analyses (Fig. 2.1). In general, doses of scN ranging from 0.1 to 0.5% resulted in significantly lower survival rate compared to the control treatment. Doses of scN ranging from 0.05 to 0.25% affected larval survival in a statistically similar manner (Table 2.1). The highest dose of scN in the diet, i.e. 0.5%, resulted in significantly lower survival than any other treatment (Table 2.1). The effect of scN on larval death appeared to be dose-dependent.

Measurements of body weight of surviving insects suggest that scN impacted the rootworm growth more dramatically during earlier developmental stages (Fig. 2.2). There was an overall significant effect of treatment on the weight of 7 day larvae (ANOVA: $F_{4, 127} = 22.0$, $p<0.0001$). Bonferroni analysis indicated there were significant differences between the control and all doses of scN ($p<0.0001$). Among doses of scN, the highest dose of 0.5% resulted in significantly lower larval weights compared to all other doses (Bonferroni 0.05%: $M = 0.12$, $SE = 0.02$, $p<0.0001$; 0.1%: $M = 0.07$, $SE = 0.02$, $p<0.03$; 0.25%: $M = 0.10$, $SE = 0.02$, $p<0.0001$). There were no significant

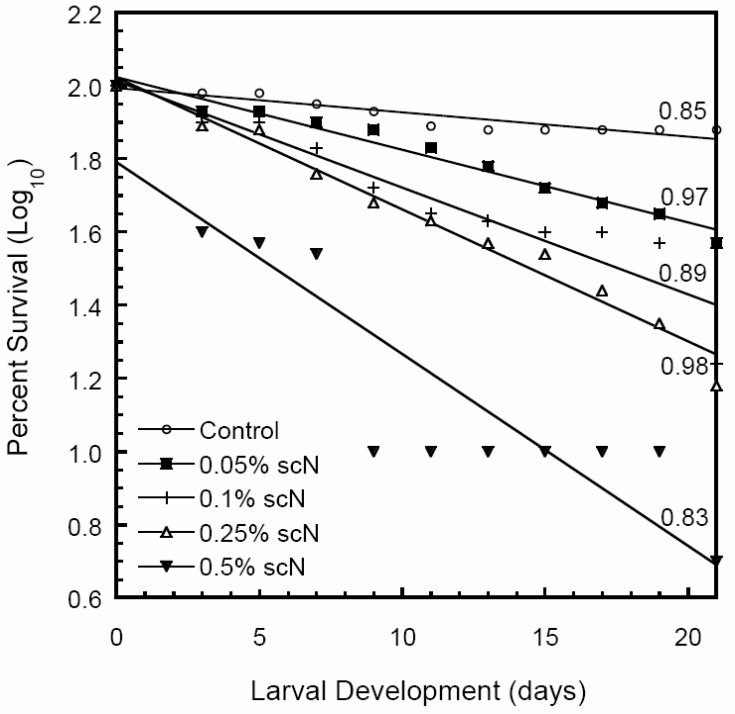


Fig. 2.1. Dietary scN increases larval mortality in a dose–dependent manner. Newly hatched southern corn rootworm larvae were reared on diet containing scN at doses indicated. Survival data were recorded as insects developed and were $\log_{10}$ transformed. The regression analysis was used to calculate regression equations and for each treatment. Linear regression R-square values were indicated in the graph (p<0.0001, respectively).

Table 2.1. Comparative analyses of linear regression lines of southern corn rootworm larval survival

Contrast	**F value**	**Probability***
Control vs 0.05%	3.97	n.s.
Control vs 0.1%	15.50	<0.001
Control vs 0.25%	25.75	<0.0001
Control vs 0.5%	148.95	<0.0001
0.05% vs 0.1%	3.78	n.s.
0.05% vs 0.25%	9.50	<0.01
0.05% vs 0.5%	104.30	<0.0001
0.1% vs 0.25%	1.30	n.s.
0.1% vs 0.5%	68.36	<0.0001
0.25% vs 0.5%	50.84	<0.0001

* n.s.: probability value >0.05, not statistically significantly different.

differences in the weights of 7 day larvae feeding on the lower doses of 0.05%, 0.1% and 0.25% scN (p>0.05).

Body weight of 11 day and 15 day old rootworms was also significantly affected by continuous feeding on scN diet (ANOVA: $F_{4,92} = 3.8$, p<0.01, and ANOVA: $F_{4,80} = 2.6$, p<0.05, respectively). In contrast to data obtained on day 7, however, Bonferroni multiple comparisons indicated that significant treatment effects at these two time points were due to the weight differences between the control and 0.05% scN diet alone (Bonferroni: $M = 0.9$, $SE = 0.2$, $p< 0.01$ for day 11 and $M = 4.3$, SE = 1.2, p<0.01 for day 15). At 0.1% dose or higher, reduction of weight gain became statistically insignificant relative to control (Fig. 2.2).

Decreased survival rates and apparent developmental delay in insects feeding on scN diet indicated that this inhibitor indeed negatively impacts southern corn rootworm larvae. Effects are most likely due to inhibition of susceptible digestive cysteine proteases, which leads to insect starvation, even death. Interestingly, as insects developed, the impact of scN on growth decreased. Although the lack of significant weight differences was confounded by low sample sizes due to high mortality and weight variation, it appears that at higher inhibitor doses, only insects with higher natural fitness under scN challenge survived and became less sensitive to its inhibition, while individuals of lower fitness did not survive the continued selection pressure. At lower doses, e.g. 0.05% however, a larger portion of the insect population could endure the stress, including less fit individuals. This survival of more susceptible insects presumably brought about the lower overall weight gain.

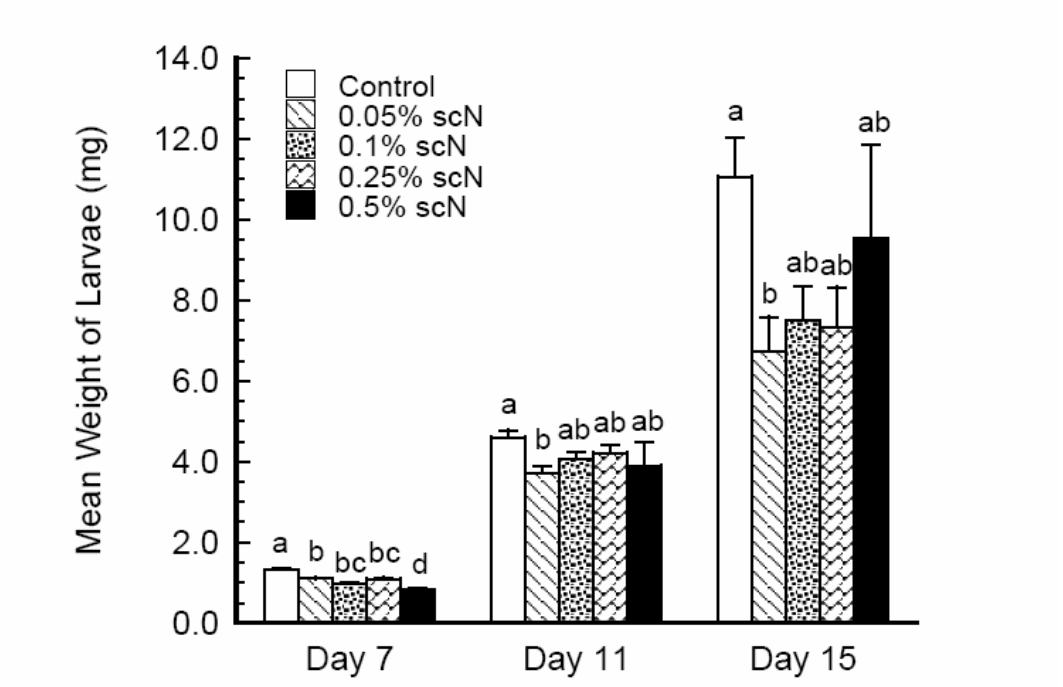


Fig. 2.2. Dietary scN substantially inhibits larval growth and development during early developmental stages. Neonate southern corn rootworm larvae were continuously reared on scN diet. For each treatment, larval weight was measured on days 7, 11 and 15 after egg hatching. Larvae fed on scN-free diet were used as the experimental control. The effect of scN was analyzed by one-way analysis of variance (ANOVA) and Bonferroni multiple means comparisons tests. Error bars indicate standard error. Means followed by the same letter are not significantly different at p=0.05.

2.3.2. Identification of scN-responsive genes from southern corn rootworm larvae

To profile gene expression from southern corn rootworm larvae in response to protease inhibitors, we constructed a subtracted (both forward and reverse) and normalized cDNA library using 2nd instar larvae fed on scN diet and on inhibitor-free diet, respectively. The advantages of subtractive hybridization method are: first, it enriches scN-responsive genes that are most relevant to the biological function we are

interested in, by excluding the majority of "housekeeping" and other unrelated genes. Secondly, the normalization step equalizes the relative abundance of cDNAs in the collected EST population, so that genes with low expression levels are also adequately represented (Diatchenko et al., 1996; Zhu-Salzman et al., 2004). The dosage of scN used for the library construction (0.1% and 0.25%) was chosen because it is high enough to cause significant mortality yet low enough to ensure sufficient insect material for mRNA extraction. The developmental stage (2^{nd} instar) was chosen because insects surviving to this stage exhibited substantially less susceptibility to scN (Fig. 2.2), suggesting that genetic machinery conditioning insect adaptation to this inhibitor is activated at or before the 2^{nd} instar.

Of the 1,920 picked clones, 1407 (i.e.73.3%) contained cDNA inserts. The average insert size, judged by PCR products, is approximately 300 bp, ranging from 100 to 500 bp. The cDNA microarrays were hybridized with probe pairs prepared from total RNAs of scN-fed and control rootworm larvae. Forty-eight clones exhibited 1.8 fold or greater changes from the control (Table 2.2). These formed 29 contigs, representing 29 genes that are differentially regulated by dietary scN. To ensure reproducibility, two replicate microarrays were conducted with separate RNA preparations from the feeding treatment. Further, selected clones showing altered expression on microarrays were subjected to northern blot analysis using RNA from a second biological replicate of the experiment (Fig. 2.3). DNA sequencing analysis indicated that they encode protein and carbohydrate hydrolases, development- and metabolism-related proteins, and genes that either had no hits in GenBank or matched genes encoding for proteins of unknown

Table 2.2. cDNA microarray identified differentially-expressed genes regulated by scN [a]

Putative function/homology	Numbers of		Ratios of signal intensity [b]		E value [c]	Accession
	Clones	Contigs	Microarray	Northern blot		numbers
1. Hydrolase						
Cathepsin L-like cysteine protease	9	2	2.37±0.68	1.79±0.82	2.00E-15	AAG17127
Cathepsin B-like cysteine protease	2	2	3.58±0.84	2.81±0.21	7.00E-49	AJ583513
Aspartic protease	1	1	2.15±0.55	2.47±0.54	2.00E-39	AAC37302
Cytosolic beta-glucosidase	1	1	0.51±0.09	0.82±0.06	1.00E-17	XP_223486
2. Development-related						
Peritrophin	1	1	2.69±0.07	2.60±0.33	6.00E-05	AAL05409
Larval cuticle protein	1	1	0.18±0.02	0.22±0.07	6.00E-10	O02388
Anterior fat body protein	1	1	0.55±0.06	n/d	8.00E-29	AAK51353
Myosin light chain 2	2	2	2.97±0.16	n/d	1.00E-09	A27270
3. Metabolism-related						
Mitochondrial rRNA gene	4	1	0.50±0.13	n/d	3.00E-53	AB104500
Phosphoribosylaminoimidazole carboxylase	1	1	0.43±0.05	0.53±0.06	1.00E-24	NP_006443
4.Others						
Acid phosphatase	1	1	0.46±0.05	0.69±0.13	1.00E-09	CAB59967
ADA2-like protein	2	1	2.19±0.23	n/d	2.00E-03	AAN35341
5. Unknown function						
Induced	18	11	1.79 to 4.78			
Suppressed	3	3	0.39 to 0.44			

a: cDNA Sequences of all clones listed in the table have been submitted to the GenBank dbEST database. Accession numbers are CO036822 to CO036850. b: Ratios of signal intensity (scN-fed vs control) were determined by cDNA microarray hybridization and confirmed by northern blot analysis as described in Materials and Methods. Shown are the mean ratios ± standard deviations of two replicate microarray slides or northern blots, respectively. scN-responsive genes are defined as having larger than a 1.8-fold change in gene expression by dietary scN. Values for genes up-regulated on microarrays are shown as >1.8, and for down-regulated are expressed as <0.56. n/d: not determined. c: BLAST searches were conducted to determine homology and the putative function of the cDNA fragments. The E value cut-off was 0.01. For those sequences that did not result in any hits using BLASTX, BLASTN and tBLASTX searches were employed.

function. Ingestion of scN increased expression of genes encoding cathepsin L- and B-like cysteine proteases and aspartic proteases in southern corn rootworm larvae (Table 2.2). Such gene regulation profiles suggest that employment of diverse proteases, both scN-sensitive and scN-insensitive, represents a strategy southern corn rootworm uses to cope with dietary inhibitors. Both cathepsin L- and B-like proteins were identified from western corn rootworm larvae, with cathepsin L-like cysteine proteases most likely the major digestive enzymes (Zhao et al., 1996; Koiwa et al., 2000; Bown et al., 2004). Although scN strongly inhibits cathepsin L-like activity (Koiwa et al., 2000), it is perceivable that increased production of scN-sensitive enzymes could outnumber the dietary inhibitor. Similar adaptive mechanisms were also found in cowpea bruchids (Zhu-Salzman et al., 2003).

cDNA microarrays identified two cDNAs encoding cathepsin B-like cysteine proteases and one encoding an aspartic protease from our subtracted library (Table 2.2, Fig. 2.3). High induction of these proteases in scN-fed larvae is both intriguing and logical, because this enzyme may be less sensitive to inhibition by scN, as the human ortholog possesses an “occluding loop” that has been shown to block the access of substrates and inhibitors (Musil et al., 1991; Illy et al., 1997). Cathepsin B-like cysteine proteases were also identified in cowpea bruchid midgut, where the protein was synthesized de novo specifically in response to scN challenge (Moon et al., 2004). In contrast, transcripts of cathepsin B-like proteases in southern corn rootworm are detectable at all larval stages. Activation of the aspartic protease gene suggested

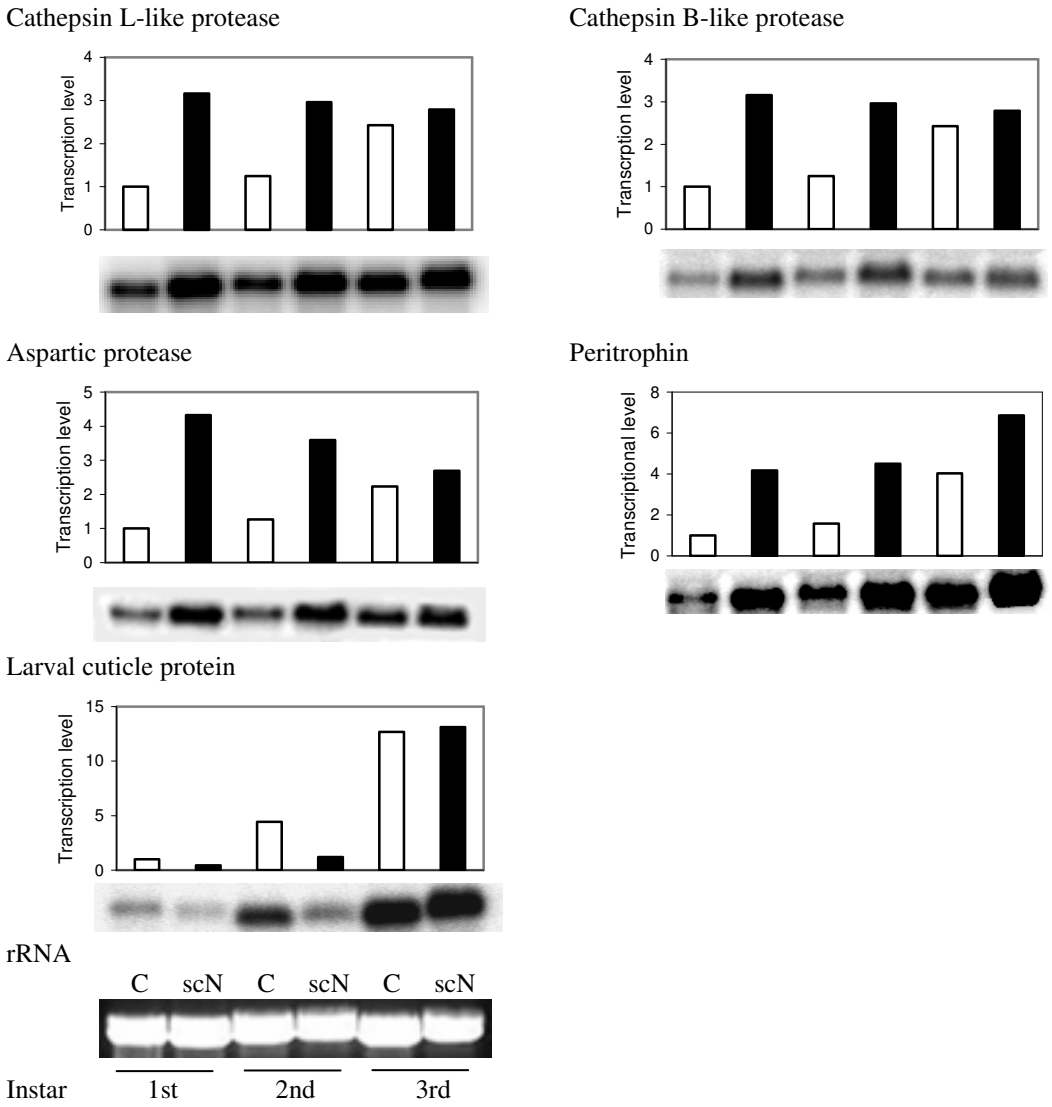


Fig. 2.3. Some scN-responsive genes are also developmentally regulated. Northern blot analysis was performed as described in Materials and methods. Total RNA (7 μg) was extracted from 1st, 2nd and 3rd instar rootworm larvae reared on control or scN diet. Respective cDNA fragments were ^{32}P-labeled and probes were used for membrane hybridization. Signals were detected by PhosphorImager and quantified using ImageQuant 5.0 software. Signal intensity from the 1st instar larvae fed on control diet was arbitrarily set at 1.0. Shown in the bar graphs are signal intensity ratios relative to the 1st instar untreated control. C: larvae were reared on scN-free diet; scN: larvae were reared on scN diet. A representative ethidium bromide-stained total RNA gel is shown to demonstrate relative RNA loading.

involvement of this additional class of enzymes to allow the insect to cope with the scN inhibitory effect. In the presence of scN in the diet, up-regulation of these types of proteases could be advantageous to insects, because cathepsin B-like enzymes are potentially less susceptible to scN and aspartic proteases are unaffected by this inhibitor.

Messenger RNA of peritrophin, an integral insect peritrophic membrane protein, was upregulated by scN (Table 2.2, Fig. 2.3). Southern corn rootworm larvae possess Type I peritrophic membrane composed of peritrophins and chitin (Ryerse et al., 1994). This membrane envelops the ingested foods along the entire midgut and plays a role in protecting the epithelial cells from mechanical damage, from invasion of microorganisms, and from digestive proteases (Tellam et al., 1999; Terra, 2001). Less effective protein degradation in the presence of scN could result in more coarse and abrasive food bolus. Presumably this causes an increased requirement and/or more rapid turnover of the peritrophic membrane synthesis to protect the midgut epithelium.

scN suppressed expression of larval development-related genes, such as those encoding larval cuticle protein, anterior fat body protein, acid phosphatase and glucosidase (Table 2.2). The biosynthesis and degradation of the cuticle protein, a component of the insect exoskeleton, is regulated by ecdysone and juvenile hormones (Willis, 1974; Andersen et al., 1995). Interestingly, suppression of the cuticle protein gene by scN occurred during the first two instars, but diminished at the 3rd instar, when insects became less susceptible (Fig. 2.3). Acid phosphatase is known as a lysosomal marker enzyme crucial to physiological cell death in insects. It is active in the guts and malpighian tubules and abundant in disintegrating tissues subjected to cytolysis, such as

silk glands and flight muscles (Yi and Adams, 2001). Also among suppressed genes were those associated with metabolism, such as 5'-phosphoribosyl-5-aminoimidazole carboxylase that catalyzes de novo purine biosynthesis pathway and is involved in DNA repair, RNA transcription and histidine biosynthesis (Patey and Shaw, 1973), and the mitochondrial 16S rRNA gene. Decreased gene expression reflected the insect's metabolic adjustment to the inhibition of its digestion. Shifting resources to synthesize proteins that counter the scN inhibitory effect presumably would slow down development.

2.3.3. Developmental regulation of scN-responsive genes

Several scN-responsive genes varied in their transcript levels at different larval stages in the absence of dietary scN (Fig. 2.3), indicating that these genes are also developmentally regulated. Indeed, the observed effect of scN on southern corn rootworm larval mortality and weight reduction diminished if the neonate larvae were reared on regular diet till the 2nd instar, and then transferred to the diet containing 0.05%, 0.10%, 0.2% and 0.25% scN ($F_{5, 39}=0.8$, $P=0.5$). Apparently, younger larvae are more susceptible to the inhibitory activity of the plant defense protein. A similar phenomenon was also observed when potato multicystatin was incorporated into the diet and fed to newly hatched and 2nd instar larvae (Orr et al., 1994).

Increased expression of digestive enzymes, whether they are sensitive to scN or not, no doubt helped the insects to cope with the inhibitory challenge. Our previous

research indicated that adaptive changes to scN in another coleopteran species, cowpea bruchid, were solely a response to the inhibitor challenge rather than a developmental event (Zhu-Salzman et al., 2003). Constitutive cathepsin L-like digestive cysteine proteases are expressed at similar levels in cowpea bruchids feeding on regular diet in all three instars. Cowpea bruchid does not express cathepsin B-like proteases unless it faces dietary challenge (Moon et al., 2004). In contrast, southern corn rootworm is capable of modulating its proteases developmentally, in addition to regulating them in response to ingestion of dietary inhibitors. Both cathepsin L- and B-like proteases are constitutively expressed in control larvae, and further enhanced in the presence of scN (Fig. 2.3).

Either direct inhibition of amino acid uptake or oversynthesis of digestive or insect adaptation-related proteins could cause a lack of essential amino acids (Jongsma and Bolter, 1997) and result in the observed impact on insects feeding on scN. The dual regulatory mechanisms presumably pose a further difficulty in using protease inhibitors for rootworm control, as a high-dose strategy has to be accompanied by the correct timing. Once insects recover from their most vulnerable neonate stage, a readjusted digestive system and strengthened physical digestive structure, whether resulting from encountering inhibitors or developmental regulation, render them insensitive to protease inhibitors.

CHAPTER III

ARABIDOPSIS VEGETATIVE STORAGE PROTEIN IS AN ANTI-INSECT ACID PHOSPHATASE—A NEW FUNCTION FOR AN OLD PROTEIN

3.1. Introduction

Vegetative storage proteins (VSP) are proteinaceous storage reserves that have been identified from numerous plants such as soybean (*Glycine max*) (Wittenbach, 1983), potato (*Solanum tuberosum*) (Mignery et al., 1984, 1988), sweet potato (*Ipomoea batatas*) (Maeshima et al., 1985), white clover (*Trifolium repens*) (Goulas et al., 2003), alfalfa (Medicago sativa) (Meuriot et al., 2004b), and in the bark of deciduous trees such as poplar (*Populus deltoides*) (Coleman et al., 1991) and elderberry (*Sambucus nigra*) (Van Damme et al., 1997). These proteins can accumulate to a high abundance, up to 50% of the total soluble proteins, in various vegetative storage organs. They act as temporary storage of amino acids that can buffer the availability of nitrogen and other nutrients. Biosynthesis and degradation of VSPs is thought to be regulated by temporary storage needs (Staswick, 1994).

The best-characterized VSPs are soybean VSPα and VSPβ (Wittenbach, 1983). They accumulate in leaves of sink-deprived soybean plants, and exist in seedling hypocotyls, developing leaves, stems, flowers and pods of mature plants as well. They are located primarily in vacuoles of the paraveinal mesophyll cells. VSP α/β do not exist in seeds, and share little sequence similarity with seed storage proteins (Staswick, 1988).

Arabidopsis thaliana VSPs share sequence similarity to the soybean VSPs, cross-react with anti-soybean VSP antibody, and are present at high levels in flowers and buds, equivalent to the expression of soybean VSP in young leaves (Berger et al., 1995). Currently VSPs are thought to serve as a transient reserve that buffers nutrient availability during plant development. In the context of plant reproduction, one can envision how depodded soybean plants need a temporary storage mechanism to sequester unused amino acids. Once new seed production begins, nitrogen and other nutrients are immediately available for seed development. Storing nutrients in various floral tissues and seed pods could help alleviate nutrient limitation during seed development. This hypothesis, however, is not supported by transgenic soybean expressing soybean antisense *VSP* gene. In this experiment, VSP expression was abolished, but contrary to what was expected, seed production was unaffected even under nitrogen-depriving condition (Staswick et al., 2001). Although the role of VSPs during seed development could be compensated for by other storage proteins, such results raised a possibility that VSPs may serve other functions beyond source-sink interaction or plant productivity.

Arabidopsis VSP transcripts are induced by mechanical wounding, jasmonic acid, insect herbivory and osmotic stresses (Berger et al., 1995, 2002; McConn et al., 1997; Utsugi et al., 1998; Xie et al, 1998; Stotz et al., 2000; Gong et al., 2001; Reymond et al., 2004), a common response shared by many genes encoding anti-insect proteins. Positive correlation between *VSP* gene expression and anti-insect activity has been observed. For instance, the *Arabidopsis fad3-2 fad7-2 fad8* triple mutant with abolished *VSP* and other

wound-regulated gene expression is highly susceptible to insect attack (McConn et al, 1997), while ethylene insensitive mutants *(ein2*, *ein3* and *etr1*) that showed higher anti-insect activity also exhibited enhanced *VSP* accumulation (Rojo et al., 1999; Stotz et al., 2000). The *Arabidopsis cev1* mutant that constitutively expresses *VSP1* and *VSP2* had higher disease resistance (Ellis and Turner, 2001). Although these observations are only correlative, it is tempting to hypothesize that *Arabidopsis* VSPs may serve as defense proteins.

Based on sequence motif analysis, *Arabidopsis* VSPs are classified as acid phosphatases of the haloacid dehalogenase superfamily, despite a lack of biochemical evidence (Thaller et al., 1998; Selengut, 2001). Phosphatases are phosphohydrolases that catalyze the dephosphorylation of a wide variety of substrates (Duff et al., 1994). Some members of the haloacid dehalogenase superfamily contain the signature motif, DXDXT, which is also conserved in many bacteria and other eukaryotes (Thaller et al., 1998; Collet et al., 1999). Plant acid phosphatases do not have absolute substrate specificity, but are important in the hydrolysis, transport and recycling of phosphate, as well as energy transfer and metabolic regulation in plant cells (Duff et al., 1994). Some plant acid phosphatases have been shown to be associated with disease resistance. Expression of *Hra28*, a putative acid phosphatase gene from *Phaseolus vulgaris*, is specifically induced by *Pseudomonas syringae*, a bacterial plant pathogen (Jakobek and Lindgren, 2002). Barley (*Hordeum vulgare*) acid phosphatase expression was increased in response to powdery mildew (*Blumeria graminis*) infection (Beβer et al., 2000).

Many plant proteins have dual or multiple roles, such as class I β-glucanases in development and defense (Reymond and Farmer, 1998). Indeed, storage proteins often possess enzymatic as well as other activities. For instance, the legume storage proteins vicilins conferred insect resistance (Yunes et al., 1998). Patatin, the storage protein from potato tubers, is a lipid acyl hydrolase (Andrews et al., 1988), whereas sporamin belongs to the superfamily of trypsin inhibitors (Yeh et al., 1997). The 32kD VSP from alfalfa is a chitinase (Meuriot et al., 2004b). Soybean VSPs have weak acid phosphatase activity (De Wald et al., 1992). The major VSP proteins in the bark of the black mulberry tree (*Morus nigra*) are jacalin-like lectins (Van Damme et al., 2002). The importance of these enzymatic activities and their relationship to the storage function are unclear. Notably however, some of these protein activities have been shown to contribute to plant defense against herbivore insects and plant diseases (Peumans and Van Damme, 1995; Radhamani et al., 1995; Creelman and Mullet, 1997; Yeh et al., 1997; Yunes et al., 1998; Van Damme et al., 2002; Meuriot et al., 2004b).

Despite the identification of signature motifs in *Arabidopsis* VSPs and abundant indirect evidence implying their defense functionality, direct evidence for biochemical and anti-insect activities is lacking. In this study, we measured the acid phosphatase activity of a recombinant *Arabidopsis* VSP2 (AtVSP2) and evaluated its effect on three insect species by incorporating the protein into their diets. Site-specific mutation indicated that the anti-insect activity of AtVSP2 is correlated with its acid phosphatase activity. To our knowledge, this is the first report that unambiguously links an *Arabidopsis* VSP to anti-insect functionality.

3.2. Materials and methods

3.2.1. Cloning and expression of AtVSP in E. coli

Ten-day old *Arabidopsis* seedlings were sprayed with 50 μM methyl jasmonate 24 hr prior to harvest. Total RNA was extracted and used for reverse transcription. The coding region of *AtVSP2* cDNA was obtained by PCR, and the PCR product was analyzed by DNA sequencing. The putative signal peptide was determined by a signal peptide-predicting software SignalP 3.0 Server (http://www.cbs.dtu.dk/services/SignalP/), as well as by sequence alignment with soybean VSP, the N-terminal sequence of which is known. The cDNA fragment excluding the putative signal peptide was then cloned in frame into the bacterial expression vector pET28a (Novagen, Madison, WI) at the *BamHI* and *XhoI* sites. The construct was transferred to *E. coli* BL21(DE3) strain (Novagen, Madison, WI) and AtVSP2 protein expression was induced by addition of isopropylthio-β-galactoside. The recombinant protein was purified through a Ni^{2+} chelate affinity column (Amersham Pharmacia Biotech, Piscataway, NJ).

3.2.2. Site-directed mutagenesis of AtVSP2 and expression of the mutant protein

Using the *pET28a-AtVSP2* construct as template, we replaced the first Asp^{119} residue in the D_{119}XDXT motif with Glu via an inverse PCR approach (Ochman et al.,

1988). Briefly, an antisense primer (5′- GGT ATC ATC TAG CTC AAA GAT CCA AAC -3′) was synthesized with the altered residue codon (underlined) located at the center flanked by complete complementary nucleotides on both sides. The sense primer (5′- CTC CTC TCT AGT ATT CCC TAC TAC GCA -3′) covered the immediate downstream sequence. Both primers were 5' phosphorylated. The PCR reaction was done as follows: 94°C for 30 s, 51°C for 1 min, 68°C for 6 min for 35 cycles. The PCR product was gel purified, diluted to 0.5 μg/ml, self-ligated, and transformed into DH5α cells. The sequence was examined to ensure the site-specific mutation. Subsequent protein expression showed that the mutated protein was insoluble. To overcome this problem, cDNA inserts (sequence altered or unaltered) were cloned into pET44a so that mutant and native AtVSP2 could be expressed as a fusion protein with NusA to improve the solubility of the expressing proteins. Both constructs were transformed into Rosetta-gami™ host cells for protein expression (Novagen, Madison, WI). The fusion proteins were purified through a Ni^{2+}-chelate affinity column (Amersham Pharmacia Biotech, Piscataway, NJ) and visualized by standard SDS-PAGE. Purified proteins were dialyzed against distilled water and lyophilized.

3.2.3. Acid phosphatase activity, pH optimum, and effects of divalent metals

Acid phosphatase activity of AtVSP2 purified from pET28, as well as Nus-AtVSP2 and mutant Nus-AtVSP2(D119E) fusion protein obtained from pET44 were evaluated using *para*-nitrophenyl phosphate (pNPP) (Sigma, St. Louis, MO) as substrate

following Hausmann and Shuman (2002). To determine the pH optimum, activity was measured in 50 mM citric acetate (pH2.5 to 4.0), 50 mM sodium acetate (pH3.5 to 5.5) and 50 mM Tris-acetate buffers (pH5.5 to 7.5), respectively. Data from overlapping pHs using different buffers were averaged. The reaction mixtures (100 μL, containing10 mM $MgCl_2$, 10 mM pNPP and *ca.* 0.03 μM purified AtVSP2 and Nus-AtVSP2, and 0.06μM Nus and Nus-AtVSP2(D119E) in respective buffers) were incubated at 37°C for 30 min, and quenched by addition of 900 μl of 1.0 M Na_2CO_3. Released *para*-nitrophenol (pNp) was detected by measuring OD_{410}, and the quantity calculated according to a standard curve.

A series of concentrations (from 0.5 mM to 50 mM) of $MgCl_2$, $CoCl_2$, $ZnSO_4$, $CaCl_2$ and $MnCl_2$ were examined for their effects on acid phosphatase activity of AtVSP2 (0.05 μM). The aforementioned reaction conditions were followed under optimal pH, previously determined to be 4.5. Absorption contributed by metals alone (i.e. reaction mixture without AtVSP2) was subtracted from the sample reading. Measurements were done in triplicate and plotted using Microsoft Excel.

3.2.4. Determination of K_m and k_{cat}

To determine apparent K_m and V_{max} values of AtVSP2 for *p*-nitrophenyl phosphate (pNPP), the rate of dephosphorylation by 0.06 μM AtVSP was measured at substrate concentrations from 1 to 100 mM. Assays were performed in 50 mM sodium acetate (pH 4.5) and 10 mM $MgCl_2$ at 37°C. Initial velocity for each substrate

concentration was calculated. Data at each concentration were collected in triplicate and were fit to the Michaelis–Menten equation ($v_0=V_{max}[S]/Km+[S]$) using the non-linear least-squares fitting analysis of KALEIDA-GRAPH software (Synergy, Reading, PA).

3.2.5. Circular dichroism and fluorescence spectra

Far-UV circular dichroism spectra of Nus-*At*VSP, Nus-*At*VSP(D119E) and Nus alone were obtained on an AVIV 62DS Circular Dichroism spectrometer (Aviv Associates Inc., Lakewood, NJ) at 25°C. The instrument scanned from 200 to 260 nm with 10 scans for each protein sample (1.1 μM for Nus-AtVSP2, Nus-*At*VSP2(D119E) and 1.5 μM for Nus). The path length was 0.5 cm. The solution for baseline spectra was 10 mM phosphate buffer, pH 7.0, the buffer in which proteins were dissolved.

Fluorescence spectra were acquired on a SLM 8100 spectrofluorometer (SLM, Urban, IL) in a 1.0-cm cuvette at 25°C. Proteins samples (0.21 μM for Nus-AtVSP2 fusion proteins and 0.53 μM for Nus) were excited at 280 nm, and fluorescence emission was scanned from 300 to 400 nm. The fluorescence contribution from the buffer was subtracted from that of the samples.

3.2.6. Insect feeding assays

To analyze the effect of AtVSP2 on insect mortality and development, artificial diets/seeds incorporated with the recombinant protein were prepared and infested in the

following manners: For southern corn rootworm assay, the artificial diet was prepared as instructed by the manufacturer (Bio-serv) using sterilizing techniques. Tetracycline and carbenicillin were added to prevent bacterial contamination. AtVSP was incorporated into the diet at various doses, dispensed into 24-well microtiter plates, and covered by parafilm to prevent drying. Nondiapausing southern corn rootworm egg masses, purchased from French Agricultural Research Inc (Lamberton, MN), were incubated at 28^{o}C and 60% RH, in the dark. For each AtVSP2 concentration, a total of 40 neonate larvae, in four replicates (10 larvae per replicate), were placed on diet. Insects were transferred every other day to new plates with fresh diets, and mortality recorded. Mean percent survival data were $\log_{10}$ transformed (Sokal and Rohlf, 1995). Linear regression analysis was performed to compare dose-mortality effects using PROC REG and user defined contrasts in SAS 9.00 (SAS Institute Inc., Cary, NC).

For cowpea bruchid, feeding procedures developed by Shade et al. (1986) were used with modifications. Decorticated cowpea seeds (California Blackeye No. 5) were milled into flour, into which AtVSP2 solutions were incorporated. Master pellets were made by injecting cowpea flour paste into the pre-cooled Teflon mold, followed by immediate freezing in liquid nitrogen as described by Shade et al. (1986). After lyophilization, the master pellets were then ground into very fine flour, from which 10 cylindrical pellets (28-mg, 4.5 mm diameter x 1.6 mm high) for each treatment were made using a mechanical hand-operated press. Each pressed pellet was then coated with gelatin by dipping the pellet in an 8% gelatin solution, and infested with one viable egg. Control artificial pellets without test proteins were made and infested in the same

manner. Within-seed developmental time was recorded, as we have previously established that it is the most reliable variable for measuring the impact of compounds on cowpea bruchids. A one-way analysis of variance test was used to analyze the data, with each infested pellet considered a replicate. Fisher's protected LSD test (p=0.05) was used for mean separation.

Effects of *At*VSP2, Nus-*At*VSP2 and Nus-*At*VSP2(D119E) on *Drosophila melanogaster* were evaluated using Kankel/White *Drososphila* medium (White and Kankel, 1978). Briefly, diet mix containing 5% glucose, 5% yeast extract, 2% yeast and 0.8% agar was boiled, and cooled to 50°C, when respective protein solutions were incorporated. To prevent fungus contamination, 0.5% of a 1:1 mixture of 83.6% propionic acid and 8.3% phosphoric acid was also added. *Drosophila* strain Canton-S was raised on a standard medium (1.48% agar, 10% corn meal, 4.12% yeast, 10% molasses, 0.8% propionic acid and 0.225% Tegosept) and transferred on an egg-collecting medium (40.5% apple juice, 5.25% glucose, 2.606% sucrose and 2.04% agar). For each protein concentration, 40 newly hatched neonate larvae, in four replicates, were transferred to the diet. The larvae were reared at 25°C and fresh diet was supplied daily. Developmental time (from egg to pupa) was recorded. Statistical analysis was carried out using SPSS for windows 11.0 (SPSS Inc., Chicago, IL). Where necessary, log transformations were conducted to normalize distributions (Sokal and Rohlf, 1995). One-way analysis of variance (ANOVA) and Bonferroni multiple means comparisons tests were performed to analyze treatment effects (Sokal and Rohlf, 1995).

3.3. Results

3.3.1. Expression of recombinant proteins

Mounting indirect evidence suggests that *Arabidopsis* VSPs may act as defense proteins against herbivore insects. To directly illustrate their biochemical properties and functional role, we first obtained an *AtVSP2* cDNA via RT-PCR from *Arabidopsis* seedlings treated with methyl jasmonate, then subcloned the cDNA devoid of the signal peptide into the pET bacterial expression system for protein expression (Fig. 3.1). A similar approach has been successfully used for recovery of soybean VSPα and nodule acid phosphatase from *E. coli* in active forms (Leelapon et al., 2004). We were able to detect recombinant proteins in the supernatant of the bacterial extract. Removal of the signal peptide apparently is critical for protein expression because no recombinant protein was detected when the signal peptide was attached (data not shown).

Existence of common signature motifs led to the recognition of *Arabidopsis* VSPs as plant counterparts of the bacterial nonspecific acid phosphatases (Table 3.1; Thaller et al., 1998). The first Asp residue of the DXDXT is thought to be the nucleophile required for catalytic phosphatase activity (Collet et al., 1998; Selengut, 2001). Once acid phosphatase activity was detected (see below), we decided to test whether catalysis was due to the aspartate nucleophile by replacing Asp^{119} (the first Asp residue in the motif) with Glu. This also allowed us to determine whether there was any correlation between the biochemical and biological activities of AtVSP2. Although

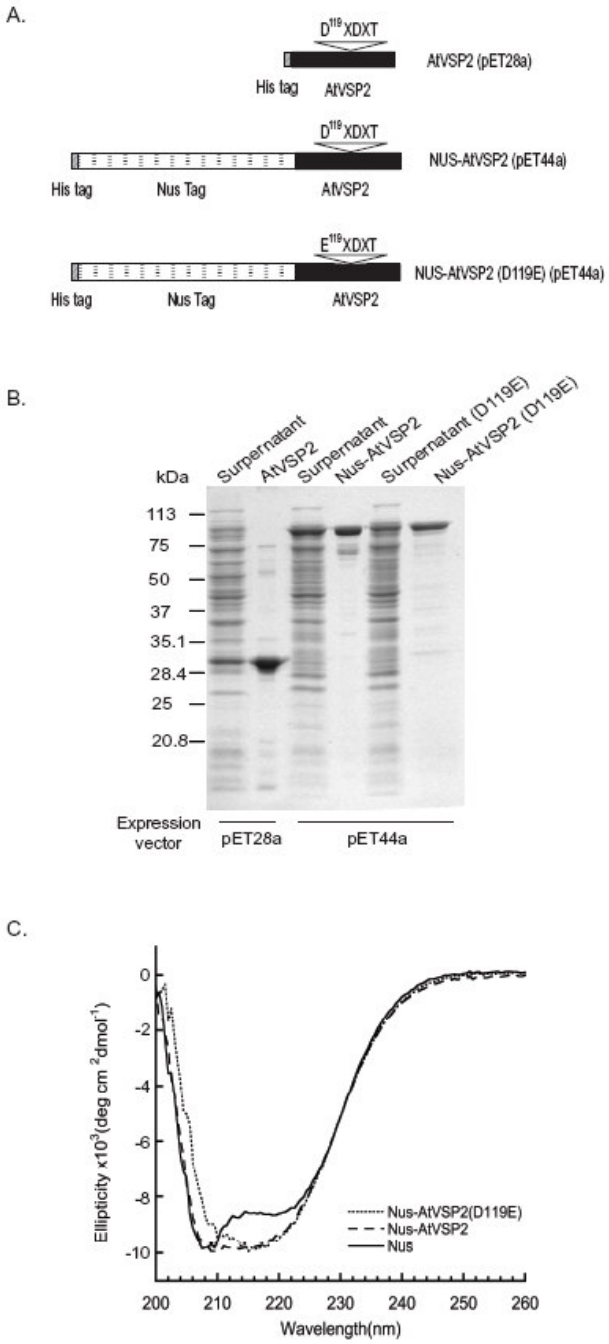


Fig. 3.1. Production of recombinant proteins in *E. coli*. (A) Constructs for expression of AtVSP2, Nus-AtVSP2 and Nus-AtVSP2(D119E). While AtVSP2 expressed via pET28a vector was soluble, mutated AtVSP2(D119E) could only be detected in the insoluble fraction. Thus both *cDNA*s were cloned into pET44a vector and proteins were expressed as fusion protein with Nus. (B) SDS-PAGE analysis of expression and purification of recombinant proteins. The supernatant of bacterial cell extract and Ni^{2+}-chelate affinity purified recombinant proteins were subjected to electrophoresis and stained with Coomassie Blue. (C) Nus tag did not interfere with AtVSP2 structure. Shown are far-UV circular dichroism spectra of Nus-AtVSP2, Nus-AtVSP2(D119E) and Nus.

Table 3.1. Signature motifs and flanking sequences conserved among selected bacterial and plant acid phosphatases and vegetative storage proteins.

Name[a]	Organism	Accession	Domain 1[b]	Domain 2	Reference
AtVSP2	*Arabidopsis*	BAA22096	115-WIF**D**L**D**D**T**LLSSIPYY--102--IVGNI**GD**QWA**D**L-20		Utsugi et al, 1996
AtVSP1	*Arabidopsis*	BAA22095	120-WIF**D**L**D**D**T**LLSSIPYY--102--IVGNI**GD**QWA**D**L-20		Utsugi et al, 1996
sAPase	Soybean	CAA11075	111-WVF**D**I**D**E**T**TLSNLPYY--105--IIGNI**GD**QWS**D**L-20		Penheiter et al., 1998
AtAPase	*Arabidopsis*	At4g25150	109-WIF**D**I**D**E**T**LLSNLPYY--102--IRGNS**GD**QWS**D**L-20		Computer annotation
tAPase	Tomato	CAA39370	105-WIF**D**V**D**E**T**LLSNLPYY--102--IVGNS**GD**QWS**D**L-20		Williamson & Colwell,1991
BCI3	Barley	AJ250282	122-WVF**D**I**D**E**T**TLSNLPYY--101--IVGNI**GD**QWS**D**I-20		Beβer et al., 2000
SIH5	Soybean	AB083030	83-WIL**D**V**D**D**T**CISNIDYY--103--IRGNV**GD**QWS**D**L-20		Hagihara et al., 2004
Hra28	Kidney bean	AY055218	113-WIL**D**V**D**D**T**CISNVSYY--103--IWGNV**GD**QWS**D**L-20		Jakobek & Lindgren, 2002
Leps2	Tomato	CAD30862	5-VVF**D**F**D**K**T**IIEVDSDN--147--RMIYL**GD**GIG**D**F-89		Stenzel et al., 2003
B-NSAP	Bacteria	P32697	65-VGF**D**I**D**D**T**VLFSSPGF--104--IRIFY**GD**SDN**D**I-40		Thaller et al., 1997
C-NSAP	Bacteria	O05471	95-IVL**D**I**D**E**T**VLDNSPYQ---97--LIMLF**GD**NLV**D**F-65		Gase et al., 1997
VSP-α	Soybean	P15490	102-FVFSI**D**G**T**VLSNIPYY--103--IVGII**GD**QWS**D**L-21		Mason et al., 1988
VSP-β	Soybean	P10743	103-FIFGI**D**N**T**VLSNIPYY--102--IVGII**GD**QWS**D**L-21		Mason et al., 1988

a. Abbreviations: Apase, Acid phosphatase; B-NSAP, Bacterial class B non-specific acid phosphatase; C-NSAP, Bacterial class C non-specific acid phosphatase; BCI3, SIH5, Hra28 and Leps2 are pathogen defense-related acid phosphatases.

b. The conserved residues were bold. Numbers indicate lengths of amino acid residues.

unmutated recombinant AtVSP2 is soluble, mutated protein AtVSP2(D119E) expressed in the pET28a vector was insoluble, thus cDNA inserts (both *AtVSP2* and *AtVSP2*(*D119E*)) were transferred to pET44a where fusion proteins with a Nus tag became soluble. Attempts to remove the Nus tag by thrombin excision were unsuccessful. Therefore, we decided to evaluate the biochemical and biological changes associated with this site-specific mutation using fusion proteins. It should be noted that the Nus tag did not interfere with AtVSP2 protein characteristics (see below).

3.3.2. Introduction of a single amino acid alteration did not compromise the protein structural integrity

To ensure that the amino acid substitution did not result in dramatic protein structural change, circular dichroism spectra were obtained between mutated and non-mutated proteins (Fig.3.1C). Results did not reveal significant differences between Nus-AtVSP2 and Nus-AtVSP2(D119E). The characteristic pattern indicative of random protein structures was not detected. Potential structural alteration was also examined by fluorescence spectra, which is based on measurements of Tyr fluorescence (Lackowicz, 1983). If substantial conformational changes occurred as the result of the mutation such that the Tyr environments were altered, a shift toward a longer wavelength of max intensity would be expected. However such a shift was not detected (data not shown), indicating that the Tyr residues in the mutant protein molecule were in relatively

hydrophobic environments, thus major structural collapse is unlikely. Such a result was expected because replacing Asp with Glu is a rather conservative change.

3.3.3. AtVSP2 is an acid phosphatase

Retention of the signature sequences in AtVSPs that are conserved for many acid phostphatases suggests that they most likely possesses this enzymatic activity and may function in phosphate production, transport and recycling. Recently, bacterial expression of soybean nodule acid phosphatase indicated the feasibility of using this system to generate active forms of this enzyme (Leelapon et al, 2004). In our experiment, AtVSP2 expressed in the pET28a vector was soluble, enabling characterization of this recombinant protein. Hydrolysis of pNPP indicates AtVSP2 is indeed an acid phosphatase. Kinetics for this substrate was determined: k_{cat}=22.0 s^{-1}, K_m=14.3 mM, and k_{cat}/ K_m is 1.5x10^3 $M^{-1}s^{-1}$. It appears that AtVSP2 specific activity was similar to soybean nodule acid phosphatase expressed in bacteria, and that both activities were significantly higher than soybean VSP activity (Leelapon et al., 2004). As expected, the pH optimum of AtVSP2 is acidic (Fig. 3.2 A). Nus-AtVSP2 expressed in the pET44a vector exhibited comparable activity to AtVSP2 produced from pET28a, despite a slight shift of optimal pH, due most likely to the presence of the Nus domain in the fusion form. Thus the Nus tag, while facilitating the solubility of the recombinant protein, did not interfere with the protein structure or enzymatic function.

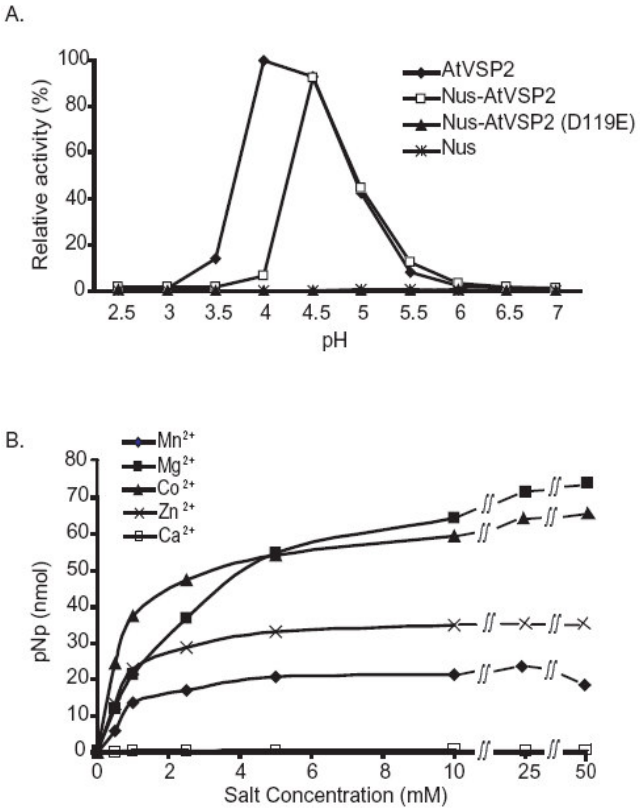


Fig. 3.2. AtVSP2 is a pH- and divalent cation-dependent acid phosphatase. (A) Reactions were buffered by 50 mM of citric acetate (pH 2.5 to 4.0), sodium acetate (pH 3.5 to 5.5) and tris-acetate buffers (pH 5.5 to 7.5). (B) Divalent cations Mg^{2+}, Co^{2+}, Zn^{2+}, Ca^{2+}, or Mn^{2+} at doses specified were evaluated for effects on phosphatase activity of AtVSP2 in 50 mM sodium acetate (pH 4.5). *p*-Nitrophenyl phosphate (pNPP) at 10 mM was used as substrate and incubated with purified recombinant proteins for 30 min at 37°C. Enzymatic activity was measured by release of *p*-nitrophenol (pNp) according to a standard curve.

It was suggested that the DXDXT motif serves as an intermediate phosphoryl acceptor and that the aspartate nucleophile was critical for catalysis (Collet et al., 1998). Consistently, a single amino acid alteration from Asp^{119} to Glu voided all acid phosphatase activity, although the protein structure remained intact (Fig. 3.2 A). Complete elimination of enzymatic activity with conservative changes at this position

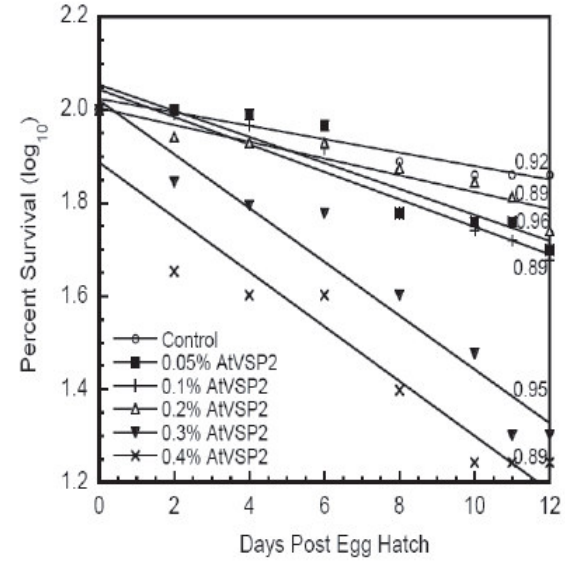
also occurred in eukaryotic Mg^{2+}-dependent acid phosphatases and phosphoserine phosphatases (Collet et al., 1998; Selengut, 2001), suggesting an essential role for this invariant residue in the signature motif.

Metal ions are known to impact enzymatic activity (Duff et al., 1994). The effectiveness of Mg^{2+}, Co^{2+}, Zn^{2+}, Mn^{2+} and Ca^{2+} as cofactors was evaluated in enzymatic assays at various doses (Fig. 3.2 B). Apparently, enzymatic activity of AtVSP2 is dependent on the presence of divalent cations. They likely function to coordinate substrate-enzyme association as illustrated in phosphoserine phosphatase (Wang et al., 2002). While Mg^{2+}, Co^{2+}, Zn^{2+} and Mn^{2+} activated acid phosphatase activity, Ca^{2+} was ineffective (Fig. 3.2 B), as were the monovalent cations K^{+} and Na^{+} (data not shown).

3.3.4. AtVSP has anti-insect activity

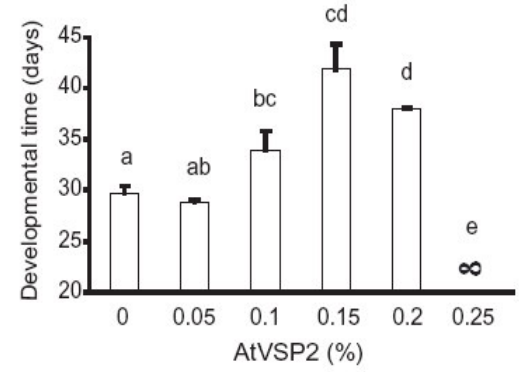
To determine whether AtVSP2 has negative effects when ingested by insects, recombinant protein was incorporated into the diets and fed to two coleopterans (cowpea bruchid and southern corn rootworm) and one dipteran (*Drosophila*). Major parts of the digestive tracts of these insects are acidic (Murdock et al., 1987; Terra and Ferreira, 1994; Edmonds, et al., 1996; Brenner and Atkinson, 1997; Bown et al., 2004), providing an environment that could promote AtVSP phosphatase activity. Developmental time is a plausible criterion for growth inhibition of cowpea bruchid (egg to adult) and *Drosophila* (neonate to pupa). However, southern corn rootworm larvae usually become

A.



Contrast	Probability	
Control vs 0.05%	>0.05	n.s.
Control vs 0.1%	>0.05	n.s.
Control vs 0.2%	>0.05	n.s.
Control vs 0.3%	<0.0001	s.
Control vs 0.4%	<0.0001	s.
0.05% vs 0.1%	>0.05	n.s.
0.05% vs0.2%	>0.05	n.s.
0.05% vs0.3%	<0.0001	s.
0.05% vs 0.4%	<0.0001	s.
0.1% vs 0.2%	>0.05	n.s.
0.1% vs 0.3%	<0.0001	s.
0.1% vs 0.4%	<0.0001	s.
0.2% vs 0.3%	<0.0001	s.
0.2% vs 0.4%	<0.0001	s.
0.3% vs 0.4%	<0.01	s.

B.



C.

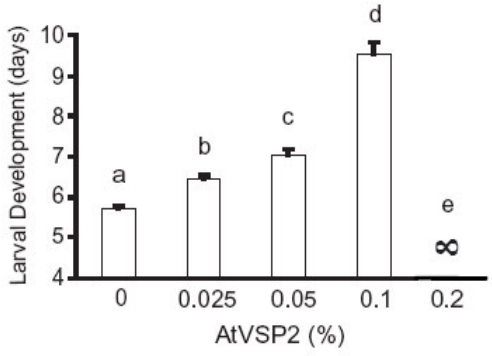


Fig. 3.3. AtVSP2 is an anti-insect protein. (A) Newly hatched southern corn rootworm larvae were reared in diet containing AtVSP2 at doses indicated. Survival data were recorded as insects developed and were $\log_{10}$ transformed. The regression analysis was used to calculate regression equations and for each treatment. Linear regression r-square values were indicated in the graph (p<0.0001, respectively). Comparative analysis, in a table format, was performed between each two treatments. Probability of 0.05 was used to determine statistical significance. s, significant; n.s., insignificant. (B) Artificial seeds containing various doses of AtVSPs were infested with viable cowpea bruchid eggs. Within-seed developmental time was recorded and analyzed by one-way analysis of variance (ANOVA). Fisher's protected LSD test (p=0.05) was used for mean separation. Error bars indicated standard error. Means followed by the same letter are not significantly different at p=0.05. ∞ indicates 100% mortality under a dose specified. (C) Neonate *Drosophila melanogaster* were reared on AtVSP2 and AtVSP2-free diet. The development time from neonate larvae to pupae were recorded. The effect of AtVSP was analyzed by one-way ANOVA and Bonferroni multiple means comparisons tests. Error bars indicated standard error. Means followed by the same letter are not significantly different at p=0.05. ∞ indicates 100% mortality under a dose specified.

infected by a fungus after rearing on artificial diet for two weeks, leading to total death prior to reaching pupal or adult stages. A bioassay method we previously developed (Liu et al., 2004) was followed for this insect. Retarded insect development and increased insect mortality was observed in all three insect species (Fig. 3.3), indicating that AtVSP2 is indeed inhibitory to their growth and development.

3.3.5. Anti-insect activity of AtVSP2 is correlated with acid phosphatase activity

The fact that the Nus domain did not appear to interfere with AtVSP2 enzymatic activity permitted us to evaluate mutant protein with the nucleophilic Asp^{119} replaced while fused with the Nus protein. To understand the AtVSP2 anti-insect mechanism, we compared the effects of Nus-AtVSP2 and Nus-AtVSP2(D119E) on *Drosophila*. Dietary Nus-AtVSP2 impacted insect mortality and development in a dose-dependent manner, comparable with AtVSP2 expressed in pET28a (Fig. 3.4). The anti-insect activity diminished in acid phosphatase-null Nus-AtVSP2(D119E) mutant protein, suggesting that the enzymatic activity is the basis for this biological function.

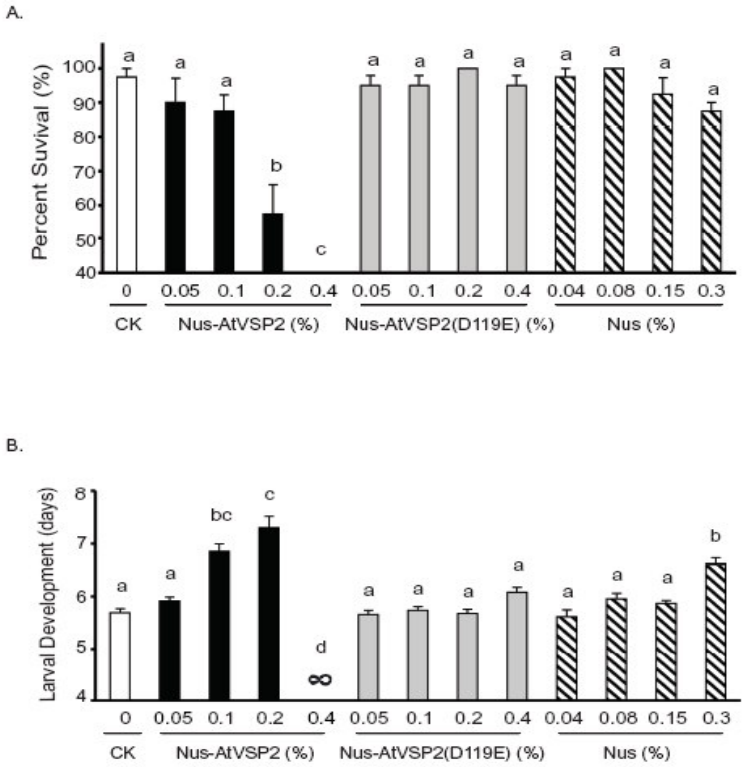


Fig. 3.4. Anti-insect activity of AtVSP2 is correlated with its acid phosphatase activity. The neonate *Drosophila* larvae were reared on diet containing Nus-AtVSP2, Nus-AtVSP2(D119E) and Nus recombinant proteins. Surviving larvae were recorded daily till pupation (A), and the development time from neonate to pupae was recorded (B). The effect of Nus-AtVSP2, Nus-AtVSP2(D119E) and Nus on the survival and development time was analyzed by one-way ANOVA and Bonferroni multiple means comparisons tests. Error bars indicated standard error. Means followed by the same letter are not significantly different at p=0.05.

3.4. Discussion

3.4.1. Temporary nutrient reservoir or phosphate metabolism?

Extensive study has been done on soybean VSPs as storage proteins. Soybean VSPs accumulate to nearly 50% of the total soluble proteins in leaves of depodded

soybean, but decline to 1% during seed fill (Wittenbach, 1982; Staswick, 1994). Such a specific function as a temporary protein storage has not been clearly established for *Arabidopsis* VSPs. Indeed, significant difference in expression levels and tissue specificity has been recognized between the so-called vegetative storage proteins from the two plant sources (Utsugi et al., 1996, 1998). *Arabidopsis VSP* genes were initially identified by random sequencing of *Arabidopsis* cDNAs, and the translated region of a partial cDNA sequence showed sequence similarity to soybean VSPs (Hofte et al., 1993). Full-length *VSP1* and *VSP2* cDNAs were later cloned from an *Arabidopsis* flower bud cDNA library (Utsugi et al., 1996), and their transcripts were shown to preferentially accumulate in flowers (Berger et al., 1995; Utsugi et al., 1996).

Classifying proteins based on short sequence motifs has received wide application as a large quantity of sequence information becomes available (Falquet et al., 2002). Motif searches are particularly useful in determining the functions of proteins encoded by unknown sequences. Many of these often lack overall sequence similarity with any proteins of known function, but it is possible to assign functions by signature motifs that exist in their sequences (Falquet et al., 2002). Undoubtedly, such motifs arise in the course of evolution due to the need to maintain protein structure in limited regions of functional importance. A new phosphatase family, the DDDD superfamily of phosphohydrolase, was recently defined by occurrence of two conserved motifs that are separated by highly variable sequence regions (Thaller et al., 1998). Plant acid phosphatases were shown, for the first time, to be closely related to the bacterial nonspecific acid phosphatases by this classification criterion. Accordingly, *Arabidopsis*

VSPs fall into this superfamily, suggesting their enzymatic functionality. Success in bacterial expression of active AtVSP2 has allowed confirmation of this biochemical property (Figs. 3.1 and 3.2). Soybean VSPs, on the other hand, were excluded from this family due to lack of the nucleophilic Asp in the DXDXT motif domain, consistent with the proposed storage functionality.

Partial sequence homology with soybean VSPs led to assignment of *Arabidopsis* VSPs as vegetative storage proteins. Illustration of phosphatase activity of the soybean VSPs further complicated the classification of the *Arabidopsis* proteins. However, the finding that soybean VSPs accounted for less than 0.1% of the total acid phosphatase activity in depodded plants while they comprised half of the total soluble proteins (Staswick, 1994) challenged the relevance of the enzymatic activity. Site-directed mutagenesis that restored the nucleophilic Asp in the first signature motif also greatly increased the acid phosphatase activity. Thus most likely it is an evolutionary necessity in soybean for the phosphatases to inactivate their catalytic activity to serve as a storage protein (Leelapon et al., 2004). Retention of the signature sequences may have supported the acid phosphatase nature of *Arabidopsis* VSPs, distinguishing them from the soybean VSPs. Substantially higher acid phosphatase activity in AtVSP2 than the residual phosphatase activity of the recombinant soybean VSP appears to support this view, although the lack of optimal natural substrates may also explain the kinetic properties. Further, when the entire open reading frames were compared, AtVSP2 showed 40.5%, 36.0% and 36.0% identities with soybean root nodule, barley and tomato acid phosphatases, respectively (data not shown). These are comparable to the

homologies between AtVSP2 and soybean VSP sequences; 40.2% identical to soybean VSPβ and 38.5% to VSPα. Partial sequence alignment and lack of sufficient acid phosphatase sequence information when *Arabidopsis VSP*s was initially identified may have biased its identification toward vegetative storage proteins (Hofte et al., 1993). Taken together, "acid phosphatases" could be a better description for the *Arabidopsis* proteins initially named as vegetative storage proteins.

Plant acid phosphatases are involved in phosphate acquisition and utilization, and their expression is subject to developmental and environmental regulation. Phosphate starvation induces *de novo* synthesis of extra- and intracellular acid phosphatases, which is thought to be one of the strategies plants have evolved to adapt to phosphate-limiting conditions (Duff et al., 1994). In *Arabidopsis*, the purple acid phosphatase AtACP5 has been well studied for its response to phosphorus starvation. Induction at mRNA and protein levels in roots as well as leaves under phosphate deficiency implies that it may function in scavenging phosphate from soil as well as recycling it within the plant (del Pozo et al., 1999). Also among phosphate starvation-induced transcripts is *AtVSP2* (Berger et al., 1995; Franco-Zorrilla et al., 2004). A common cis-element motif in the promoter regions recognized by the phosphate-starvation responsive transcription factor PHR1, is shared by this group of genes (Franco-Zorrilla et al., 2004). In contrast, the PHR1-binding motif is lacking in the soybean *VSP* promoter, rather its response to phosphate-deprivation is regulated by homeodomain-leucine zipper proteins (Tang et al., 2001). Other evidence shows that *Arabidopsis VSP* regulatory profiles are more similar to soybean *VSP* than *AtACP*. For instance, ABA, JA, salt, water deficit and wounding

stresses induce expression of soybean and *Arabidopsis VSP*s, but not *AtACP5* (del Pozo et al., 1999). The role of *Arabidopsis* VSPs in phosphate regulation needs to be further investigated.

3.4.2. Defense: one possible biological function in plants for AtVSP

Despite the overall sequence similarity between *Arabidopsis* and soybean VSPs, differences in tissue specific expression are prominent. *Arabidopsis* expresses VSPs predominantly in the flower, and very little in leaves (Utsugi et al., 1998). The contrasting expression patterns can be interpreted by the assumption that *Arabidopsis* VSPs may have other functions beyond being a temporary protein reserve. A number of plant acid phosphatases have been implicated in resistance to pathogens and nematodes (Williamson and Colwell, 1991; Beber et al., 2000; Jakobek and Lindgren, 2002; Petters et al., 2002; Stenzel et al., 2003). They appear to be induced by different regulatory pathways; while barley acid phosphatase was induced by the salicylic acid mimic 2,6-dichloroisonicotinic acid and jasmonic acid, potato acid phosphatase responded only to intact bacterial organisms. Induction of gene expression in response to insect herbivory and correlation between increased *Arabidopsis* VSP expression and enhanced plant resistance to Egyptian cotton worm (*Sponoptera littoralis*) (Rojo et al., 1999; Stotz et al., 2000; Berger et al., 2002) suggested a possible role in defense again herbivore insects. This concept has been confirmed here by the feeding experiments that clearly illustrated AtVSP2 toxicity to insects.

High expression of VSP proteins in flowers most likely represents a mechanism *Arabidopsis* uses to protect its reproductive structures. Flowers, while being attractive to pollinators, may also be visual signals for herbivorous insects. Thus it is not surprising that plants devote resources to synthesize defensive compounds to guard their reproductive organs and developing seeds. One illustration is via the floral UV pigments, flavonoids and dearomatized isoprenylated phloroglucinols, from *Hypericum calycinum* (Gronquist et al., 2001). These UV pigments are responsible for forming UV patterns on flowers visible to insects, to increase attractiveness to pollinators. Meanwhile, they are also toxic compounds to caterpillars. Therefore, they appear to play dual attractive and protective functions in the plant. Interestingly, they accumulate to very high concentrations in anthers and the ovary wall of the flower and are expressed at low levels in leaves (Gronquist et al., 2001).

It is intriguing that a very similar tissue-specific pattern also occurred in *Arabidopsis* VSP expression (Utsugi et al., 1998). As sink tissues of the plant reproductive phase, floral organs and developing seeds draw significant amounts of sugar, amino acids and ions from source tissues (Meuriot et al., 2004a), and are thus natural targets for herbivorous insects. In leaf tissue, VSP expression can be rapidly induced by wounding, jasmonate or insect feeding (Berger et al., 1995, 2002; Utsugi et al., 1998; Stotz et al., 2000; Reymond et al., 2004). *AtVSP2* also responded to osmotic stresses exerted by drought and salt treatments (Gong et al., 2001). Since water-deficit stress in plants tends to cause increased infestation by insects (Flint et al., 1996), activation of *VSP* may also strengthen plant defense aside from a possible role in source-

sink regulation. Developmental regulation in a tissue-specific manner and response to wounding and insect herbivory in the otherwise low VSP expressing leaf tissue suggested that VSP proteins belong to the defense arsenal that plants rely on to ensure protection of offspring.

Although toxicity to insects appears to be related to enzymatic activity, it is unclear whether AtVSPs target specific substrates. Acid phosphatases are generally considered to lack substrate specificity, however some specificity has been shown (Duff et al., 1994). Screening insect mutant lines that have altered susceptibility to AtVSP2 may help reveal the insecticidal mechanisms of plant acid phosphatases such as *Arabidopsis* VSPs.

CHAPTER IV

GENERATING ATVSP-RESISTANT *DROSOPHILA* MUTANTS BY USING *P*-ELEMENT MEDIATED MUTAGENESIS

4.1. Introduction

Drosophila melanogaster has been used as a model organism for understanding eukaryotic biology and genetics over the past 100 years. The advantages of *D. melanogaster* are: (i) it is easy and cheap to rear in the laboratory; (ii) it has a ten-day generation time and produces many progeny; (iii) there are only four chromosomes and a relatively small genome (165 Mb). The completed genome sequence is available; (iv) it provides a wealth of external features (visible markers), which can be altered by mutations and are scorable under the stereomicroscope; (v) most importantly, the *P*-transposable element offers a fabulous way to manipulate the fly genome: targeted mutagenesis of desired genes, incorporation of transgenes into the genome; identification of interesting loci by enhancer trapping (Rubin and Spradling, 1982; Kaiser and Goodwin, 1990; Kaiser, 1993).

P-elements are thought to have entered the *D. melanogaster* population less than 100 years ago by horizontal transfer from other *Drosophila* species. They have since spread to most wild and laboratory populations (Ryder and Russell, 2003). They were first recognized as factors in *P* strains that caused hybrid dysgenesis (a syndrome that includes mutation, sterility and male recombination) in crosses between male *P* strains

and female *M* strains (Kidwell et al., 1977). *P* –elements can be divided into two groups: autonomous elements, which encode their own source of the transposase needed for mobilization, and non–autonomous elements that need an external source of transposase in order to move. Non-autonomous elements occur either as natural mutations, produced as a result of internal deletions, or in the form of the engineered constructs widely used in laboratory studies. The wild-type autonomous *P*-element is 2.9 kb in size and contains a four-exon transposase gene and a number of inverted repeats. In order to transpose, all *P*-elements must have intact 31-bp perfect inverse terminal repeats and 11-bp subterminal inverted repeats. The repeats are the site of action of the transposase. *P*-element transposition is naturally restricted to the germline because the splicing of the intron between exons 2 and 3 of the transposase is inhibited in somatic cells by a splicing repressor protein (O'Hare and Rubin, 1983; Amarasinghe et al., 2001). In the somatic cell, the retention of the third intron results in a truncated transposase protein that acts as a repressor of *P*–element mobility (Ksress and Rubin, 1984). This truncated repressor is also responsible for the fact that, in wild type strains, *P*-element mobility is restricted to crosses between M strain females and *P* strain males, since *P* strain females pass on the repressor protein through the cytoplasm of their eggs (Laski et al., 1986; Misra et al., 1993).

Early attempts to utilize the *P* element were involved in mobilization of large numbers of natural *P* elements (Kidwell, 1985). Lines obtained in this manner were difficult to use for genetic or molecular studies since extensive out-crossing and recombination experiments were required to eliminate *P* elements other than the one

responsible for the mutation of interest. Consequently, the development of a means for mobilizing a single marked *P* element in each mutagenized strain was a considerable step forward (Cooley et al, 1988). The essential nature of mobilization of a single *P* element is to use two separate *P* elements. The first is a genetically marked *P* element that is defective in producing transposase but contains the ends required for transposition. The second is a *P* element with functional transposase activity but lacking inverted repeats for its own transposition (Robertson et al., 1988). Transposition of the marked *P* element is then initiated by crossing flies that carry only the marked *P* element to those that harbor only the transposase. Insertions generated by this scheme are recovered in flies lacking transposase activity and are therefore genetically stable.

P element mutagenesis efforts have culminated in the Gene Disruption Project, a consolidated effort to mutate every gene in the Drosophila genome (Spradling et al., 1995; Spradling et al., 1999; Bellen et al., 2004). Over the past dozen years, various projects have generated collections of single *P*-element mutants. By 1999, the Berkley Drosophila Genome Project (BDGP) gathered 1045 strains using *P* elements, such as *PZ[ry], P-lacW* and *Phsneo* (Spradling et al.,1999). Until 2004, the BDGP gene collection was up to 7140 strains (5362 genes) by using both *P*-elements (e.g. *P{GT1}*, *P{EPgy2}*) and *piggyBac* elements (e.g. *PBac{5HPw[+]}*) (Bellen et al., 2004). In FlyBase, 9161 different *P* element alleles are listed (The FlyBase Consortium, 2002).

However, whether the goal of obtaining an insertion in every gene is achievable with the *P*-element is a matter of some debate due to the presence of 'hot spots' (sites that were hit at extremely high frequency) and 'cold spots' (sites that have never been hit

to date) in the fly genome. There are two classes of genes that act as transposon 'hotspots'. The first class comprises genes or loci that evidently possess favorable chromatin accessibility, DNA target sequences, or bound proteins that mediated high-efficiency association with a freely diffusing transposition complex. Euchromatic sites are favored over heterochromatic sites; interbands appear to be favored over bands in polytene chromosomes. It is also notable that there is a marked tendency of *P*-elements to integrate at the 5'-end of genes (Tsubota et al., 1985; Spradling et al., 1995). Liao et al. (2000) argued that the structural feature of the target DNA plays a more important role for tranposon integration than a specific sequence of nucleotides. Their results imply that interaction of *P* transposase with the *P* insertion site is facilitated by both DNA structural features and degenerate patterns of hydrogen binding sites in the major groove of the DNA double-strand helix. The second class of 'hotspots' are highly screen-dependent (*i.e.* dependent on the original site and type of *P* element construct). They are likely to be physically proximal to the starting transposon used in the screen (O'Hare and Rubin, 1983, Tower et al., 1993). The structure of a constructed *P* element may also influence its own transposition (Bellen et al., 2004).

Although the *P*-element mutagenesis results in biased interruption of the fly genome, it is still widely used for dissecting gene functions involved in diverse biological and physiological processes *in vivo*. It has been successfully used to identify genes involved in the development of embryonic peripheral nervous system (PNS) (Kania et al., 1995), isolate the targets of anesthetics(Gamo et al, 2003), and reveal the

crucial genes involved in overgrowth as well as other complex phenotypes (Torok, et al. 1993).

We generated a mutant population that can be screened directly for AtVSP resistance using single-copy *P*-element mediated mutagenesis. We have isolated putative mutant stocks and the corresponding revertants. Evaluation of their sensitivity to AtVSP is currently under the way. The molecular and genetic analysis of these mutants will potentially further our understanding of the mechanism of AtVSP anti-insect activity.

4.2. Materials and methods

4.2.1 Strains

Strain w; PJ29 has a single copy of a *P* transposon (*P-lacW*) that encodes a lacZ reporter gene and a mini-white gene (w^{+mC}) that provides a visible marker. A map of *P-lacW* is presented in Figure 4.1. The transposable portion of *P-lacW* contains a bacterial origin of replication and the β–lactamase gene coding for ampicillin resistance at its 3' end. This feature permits easy cloning of DNA flanking the insertion site of *P-lacW*. The mini-white gene has been inserted into the middle of *P-lacW* (Bier et al., 1989). The strain used to supply transposase activity is w;Sp/CyO;SbΔ2-3/Tm6. It contains a modified *P* element that is missing the third intron (Δ2-3) has high levels of transposase activity and is capable of mobilizing other elements, yet is itself remarkably stable (Robertson et al., 1988). The SbΔ2-3 transposase-producing allele was marked with the

dominant stubble bristle marker on the third chromosome over the TM6 balancer. The yw strain has white eyes, yellow body and bristle. The strains w;ana{1}/CyO and yw;bnl{P1}/TM3·Sb·Ser·y^+ have white eyes and provide the second and third balancer, respectively. All flies were maintained and mated on standard yeast-cornmeal-agar medium and all experiments were performed at 25ºC.

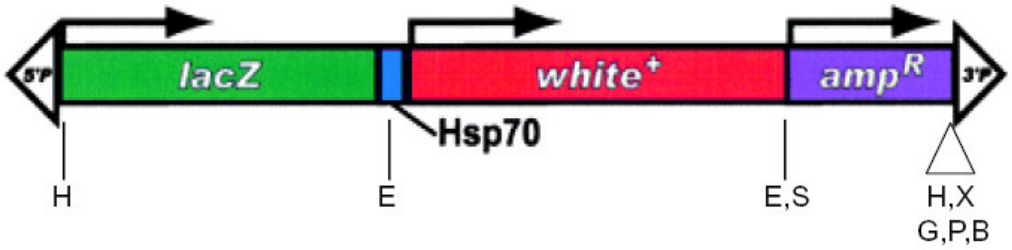


Fig. 4.1. Features of *P-lacW*. The transposable portion of *P-lacW* is 10.6 kb long. At the 5' end of the transposon is the *P*-element transposase –lacZ fusion (pTps-lacZ), which is followed by the mini-white gene (in the same orientation for transcription). At the 3' end of *P-lacW* there is the plasmid origin of replication (Origin) and the β–lactamase gene (amp^R). The plasmid sequences are bounded by two linker sequences containing EcorRI and SacII sited in one and unique sites for XbaI, PstI, BglII and BamHI. These restriction sites permit rescue of genomic sequences in either site of the integration site. Restriction enzymes abbreviations: (H) HindIII; (E) EcorRI ; (S) SacII; (X) XbaI;(P) PstI;(G) BglII and (B) BamHI. Hsp70: heat shock protein 70. The fusion protein does not contain Hsp70, since it is preceded by the lacZ translation stop.

4.2.2. *P*-element mutagenesis and AtVSP resistance screening

The crossing scheme is shown in Figure 4.2. Female or male homozygous w;PJ29 having the *P-lacW* insertion were crossed to w;Sp/CyO;SbΔ2-3/Tm6 male or female carrying transposase Δ2-3. This cross yields 'jumpstarters' that carried both the *P-lacW* and the Δ2-3element, in which the *P-lacW* gets mobilized and jumps into new

insertion sites. Because a lack of the intron in the Δ2-3 *P* element causes expression of transposase in all tissues, flies that carry both *P-lacW* and Δ2-3 have eyes with patches of colors mixed with white areas, a phenomenon known as somatic variegation (Fig.4.2).

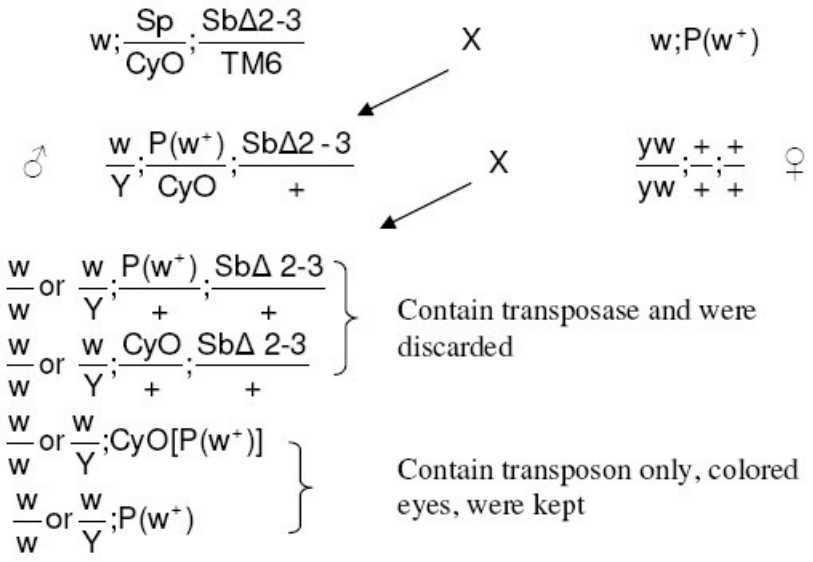


Fig. 4.2. Mating scheme for *P* element mutagenesis. Symbol abbreviations: w=white-eye color; Sp=sternopleural, extra hair; CyO=curly wings; Sb=stubble hair; Tm6=balance, (keep SbΔ2-3 on same chromosome); Y=Y chromosome; y=yellow body and hair; +=wild type. P(w^+)= *P-lacW* transposon carrying w^+ marker gene. Low case letters indicate recessive allele and capital letter dominant.

The male jumpstarters were crossed to homologous yw virgin females to generate offspring. Ten thousand newly hatched larvae of F2 progeny were collected and raised on a diet containing 0.3% AtVSP. The survivors were reared as individual stocks. Only those that no longer contained transposase SbΔ2-3 were retained. These putative AtVSP-resistant mutants were subjected to secondary screening. Approximately 100 progeny generated from crossing the putative AtVSP-resistant mutants to yw strain were reared

on diet containing 0.3% AtVSP. The number and phenotype of survivors were recorded. The progeny segregation from the crosses also indicated whether the *P-lacW* is located on the sex-chromosome.

4.2.3. Establish AtVSP-resistant mutant strains

The confirmed AtVSP-resistant mutant strains were crossed to w;ana{1}/CyO (contains 2nd chromosome balancer) and yw;bnl{P1}/TM3·Sb·Ser·y^+ (contains 3rd chromosome balancer) (Fig.4.3) . The insertions were balanced to suppress cross-overs. Segregation results could suggest which chromosome the *P-lacW* is located on; meanwhile permanent stocks were obtained.

4.2.4. Verification of insertions responsible for the AtVSP resistant phenotype

To ascertain whether the resistant phenotype was caused by the *P-lacW* insertion, the *P* element was remobilized to induce precise excision. Females from the mutant strains were crossed to w;Sp/CyO;SbΔ2-3/Tm6 (Fig. 4.4). "Jumpstart" males from the progeny were crossed to w;*P(w^+)*/CyO females. The curly, non-stubble, white-eyed progeny were individually crossed to w;*P(w^+)*/CyO. The white-eyed curly lines were propagated and tested on AtVSP diet for sensitivity.

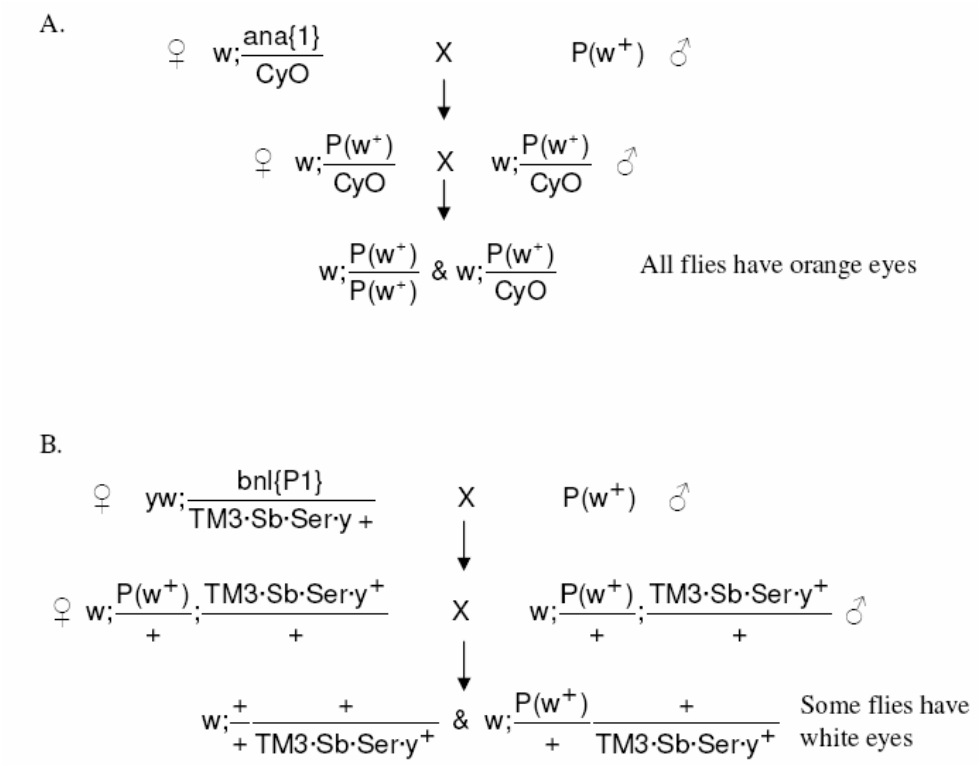


Fig. 4.3. Balancing scheme of AtVSP-resistant mutants. It also helps to identify the location of transposon *P-lacW*. A. CyO balancer is on second chromosome; B. TM3·Sb balancer is on third chromosome.

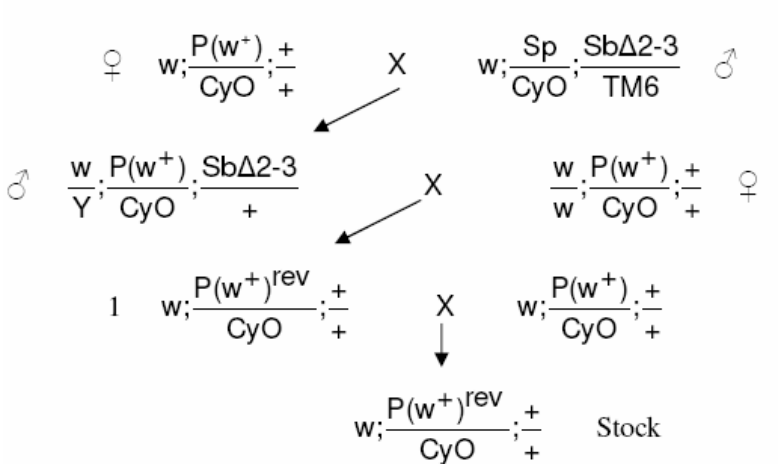


Fig. 4.4: Isolation of revertants by remobilizing the *P-lacW*. $P(w^+)^{rev}$ represents a revertant chromosome with precise excision of the *P-lacW*. See text for details.

4.3 Results

4.3.1. Isolate the AtVSP-resistant mutants and establish the balanced mutant stocks

Eight survivors were isolated from 10,000 larvae (from the cross of jumpstart males to yw females). Three out of the eight flies having stubble hair and variegated eyes were discarded because the presence of transpoase makes the *P* element unstable. The other 5 survivors (1 female and 4 males) contain no transposase but transposon P(w^+) (*P-lacW*). These putative AtVSP-resistant mutants were crossed to yw flies and their progeny were subjected to the second screening. Among these, two mutants (1 female and 1 male) did not survive (indicative of escapes). One male mutant did not produce any progeny after crossing since the eggs never hatched. This mutant was thus discarded. One mutant with 6 survivors out of 100 progeny on the 0.3% AtVSP diet was termed as VR1 (AtVSP Resistant mutant). The other one produced 4 survivors out of 100 and was named as VR2. The genotype of both mutants can be described as w;P(w^+).

When male VR1 and VR2 were crossed to yw female, not only the female but the male progenies showed orange eyes, which suggested that the P-*lacW* transposon was not on the sex chromosome. Then VR1 and VR2 were crossed with flies having second and third chromosome balancers, w;ana{1}/CyO and yw;bnl{P1}/TM3·Sb·Ser·y^+. They produced w;CyO [P(w^+)]* and w;TM3·Sb [P(w^+)] flies (*brackets were used because the location of P(w^+) is unknown until this step). All flies from w;CyO [P(w^+)] self crossing have orange eyes and they are either curly winged or straight winged. This

observation indicated the *P-lacW* insertion was on the second chromosome. The two kinds of flies are P(w^+)/P(w^+) and P(w^+)/CyO. No CyO/CyO and white-eyed flies were observed because two copies of balancer make the flies too weak to survive. The straight-wing flies (i.e. homozygous for *P-lacW*) have darker eyes than those of curly flies (i.e. heterozygous for *P-lacW*). We observed the white eye flies in the progeny of w;TM3·Sb[P(w^+)] self crossing, indicating that *P-lacW* is not on the third chromosome. Thus, VR1w; P(w^+)/CyO and VR2w; P(w^+)/CyO balanced stock were established. The progeny of VR1w; P(w^+)/CyO and VR2w; P(w^+)/CyO were reared on 0.3% AtVSP diet again. We obtained 8 survivors out of 100 for VR1 and 4 out of 100 for VR2. Among the survivors, there were no biased straight-wing flies. The homozygous (w^+/ w^+) progenies of VR1 and VR2 were reared on the AtVSP diet. They showed lower resistance compared with the progenies of the mixture of heterozygous and homozygous (Table 4.1). VR1 showed a little higher resistance to AtVSP than VR2 in these three independent bioassays using homozygous or mixture of heterozygous and homozygous individual.

Table 4.1. Survivors of putative AtVSP-resistant mutant strains

Strain	Heterozygous and homozygous Non-balanced	Heterozygous and homozygous CyO balanced*	Homozygous
VR1	6/100	12/100 ; 8/100	3/100
VR2	4/100	7/100 ; 4/100	1/100

* Two numbers in this column came from two independent bioassays.

4.3.2. Reversion of the *P*-induced mutations

To directly correlate insertions with mutant phenotype, i.e. AtVSP resistance, we carried out reversion tests. The VR1 and VR2 strains were reexposed to transposase. Reversion of the phenotype was detected by the appearance in a subsequent generation of AtVSP-sensitive adults having white eyes. This is a direct demonstration that the AtVSP-resistant phenotype is due to the insertions. Of course only precise excision can be correlated to reversion of the phenotype uniquely confirming the given insertion mutation as the direct cause of the phenotype. Imprecise excision allows the generation of new allele that can be useful in phenotype analysis.

This part of work will be carried out.

4.4. Discussion

We carried out the dominant mutant screening such that the only a limited number of genes in the fly can be detected. The total number of genes in *Drosophila melanogaster* is 14015 (Flybase) while the known dominant genes are approximately 1800. The euchromatin of *Drosophila* X chromosome contains 2182 predicted protein coding genes (both recessive and dominant genes), which is around 20% of the total gene numbers (Adams et al., 2000). Thus approximately 4000 genes could be potentially detected if they indeed confer AtVSP resistance through our screening approach. However, not all these genes can be interrupted by the *P* elements because of the

presence of coldpots. It was reported that each chromosome contains 5 to 10 hotspot loci that accumulate as many as 30 to 50% of the *P* element insertions in any particular screen (for example, see Kania et al., 1995; Salzberg et al.,1997; Spradling et al.,1999). Therefore the number of the genes that can be targeted by *P* element in our screening would be dramatically decreased.

The two VR mutants we obtained showed a low level of resistance to AtVSP. One possible explanation is that the insecticidal activity of AtVSP relies on several targets in *Drosophila*. AtVSP is one of the acid phosphatases, important in the hydrolysis and mobilization of phosphate from extracellular phosphomonoesters for plant nutrition, but are relatively non-specific (Duff, 1994). Theoretically, any phosphorylated molecule could serve as a substrate. The single *P*- element in the VR mutant only alters one AtVSP target, while other targets are still available for AtVSP. Thus, the mutants could show a lower level of resistance.

Assuming multiple AtVSP targets exist, resistance could be treated as a quantitative trait that may be shaped through many genes or their interactions. We would expect to observe various level of resistance when the *P* element disrupted other AtVSP targets. The resistant levels of the two VR mutants are slightly different, suggesting different contributions to resistance. Isolating more mutant lines and characterizing genes that regulate the resistance response may help find more AtVSP targets. Some statistical analysis and algorithm modeling for the isolated *P*-insertion lines will assist to assess the magnitude of individual mutational variance for AtVSP resistance (Harbison et al., 2003). Screens for quantitative effects of *P* element insertions have been

successful in identifying novel loci affecting metabolism (Clark et al., 1995), sensory bristle number (Lyman et al., 1996; Nogra et al., 2003) and olfactory behavior (Anholt et al., 1996).

4.5. Future studies

Cloning insertion site DNA, locating loci and identifying genes of AtVSP-resistant mutants.

Once the sensitivity of VR1 and VR2 revertants to AtVSP is confirmed by bioassays, the loci or genes disrupted by the *P* element in AtVSP-resistant mutants will be sequenced. The genomic DNA fragments flanking the *P*-element will be isolated by inverse PCR. Generally, DNA will be extracted from the AtVSP-resistant lines VR1 and VR2, and then digested to completion with a restriction endonuclease. Restriction sites using for the 5' flanking region are *EcoR*I and *Sac*II and the 3' region are *Xba*I, *Pst*I, *Bgl*II and *BamH*I (Fig. 4.1). The restricted DNA will be ligated under diluted conditions to circularize the restriction fragment containing the *P* element and the flanking genomic DNA. The circularized products will be used as templates for PCR. PCR products that contain the flanking genomic DNA fragments will be sequenced and a homology search will be carried out using genetic databases (http://www.fruitfly.org/ or http://flybase.bio.indiana.edu/). The loci will be located and putative genes will be

identified. All the protocols for genomic DNA preparation, digestion, inverse PCR and sequencing are available in http://www.fruitfly.org/about/methods/inverse.pcr.html.

CHARPTER V

SUMMARY

In the course of their evolution, plants have developed a multitude of defense mechanisms against herbivorous pests, including various defense-related proteins. The genes that encode plant defense proteins, such as protease inhibitors, α-amylase inhibitors, lectins and chitinases, have been proposed for use in designing plants resistant to pests. However, mounting evidence indicates that insect counterdefense mechanism are a major obstacle to the manipulation and utilization of these plant defense proteins. To design more effective and longer-lasting plant defense, we must understand the mechanisms utilized by insects to counteract them. We used southern corn rootworm, a notorious insect pest in corn and peanut, and a soybean cysteine protease inhibitor, soyacystatin N (scN), to uncover the insect adaptation mechanism. The results indicated that southern corn rootworm larvae exhibited increased mortality and reduced growth rate when fed on a diet containing scN. scN impacted mortality in a dose-dependent manner, and its effect on insect growth was more severe at early developmental stages. Insects that survived from continuous exposure to the inhibitor at doses ranging from 0.1 to 0.5% had less reduction in body weight during later developmental stages. This insensitivity as insects develop was not observed in the insect group fed on diet containing 0.05% scN, the lowest dose tested. Thus individuals that survived the higher dose treatment may have higher fitness under dietary inhibitory challenge. Subtractive hybridization and cDNA microarray analyses identified 29 transcript species responsive

to scN. Southern corn rootworm larvae over-expressed cysteine and aspartic proteases to compensate for inhibition of digestion. Induction of a peritrophin gene suggested that strengthening the peritrophic membrane may play a role in coping with protease inhibitors. scN down-regulated genes encoding proteins involved in insect metabolism and development, reflecting the insect's ability to reallocate resources to prioritize its defense response. Furthermore, protease and the peritrophin genes were also developmentally regulated, which may explain the lower toxicity in older larvae than in neonates when first encountering dietary scN. This study indicated that multiple regulatory mechanisms of counter defense-related genes may allow insects to evade the effect of plant defense proteins, and impose an obstacle to biotechnology-based insect control.

Vegetative storage protein is one of the final products in the jasmonate signaling pathway that is essential for insect defense. The close correlation of VSP induction with jasmonate treatment may suggest a potential role of VSP in plant defense. Indirect evidence suggested that *Arabidopsis* vegetative storage protein (VSP) plays a role in defending its host against herbivorous insects. To test this hypothesis, we expressed an *Arabidopsis VSP2* (*AtVSP2*) in *E.coli*, a wound-, jasmonate- and insect feeding-induced gene. The recombinant protein exhibited phosphatase activity in the acid pH range. When incorporated into the diets of three coleopteran and dipteran insects, which have acidic gut lumen, recombinant AtVSP2 significantly delayed development of the insects and increased their mortality. To further determine the biochemical basis of the anti-insect activity of the protein, the nucleophilic Asp^{119} residue at the conserved DXDXT

motif was substituted by Glu via site-directed mutagenesis. This single amino acid alteration did not compromise the protein's secondary or tertiary structure, but resulted in complete loss of its acid phosphatase activity as well as its anti-insect activity. Collectively, we conclude that AtVSP2 is an anti-insect protein and that its defense function is correlated with its acid phosphatase activity.

Although toxicity to insects appears to be related to enzymatic activity, it is unclear whether AtVSPs target specific substrates. Screening *Drosophila* mutant lines that have altered susceptibility to AtVSP2 may help reveal its insecticidal mechanisms. We carried out *P*-element mediated mutagenesis to generate the mutants that were able to be screened directly. Two AtVSP resistant mutants were obtained. They showed low level of resistance. Revertant analysis is underway to confirm whether the *P*-element insertion is likely to be responsible for the observed mutant phenotype. If the analysis shows that *P* element insertion causes the resistance, the genomic flanking DNA fragments will be isolated by inverse polymerase chain reaction (IPCR). The results will further our understanding about the mechanism of anti-insect activity of AtVSP.

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