Does Gamma-Aminobutyric Acid Function as a Plant Resistance Mechanism Against Phytophagous Insect Activity?

by

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1. Abstract

Gamma-aminobutyric acid (GABA) is a ubiquitous non-protein amino acid synthesized via the decarboxylation of L-glutamate in a reaction catalyzed by the cytosolic enzyme L-glutamate decarboxylase (GAD). In animals it functions as an inhibitory neurotransmitter. In plants it accumulates rapidly in response to various stresses, but its function remains unclear. The hypothesis that GABA accumulation in leaf tissue may function as a plant resistance mechanism against phytophagous insect activity was investigated.

GABA accumulation in response to mechanical stimulation, mechanical damage and insect activity was demonstrated. In wt tobacco (Nicotiana tabacum cv Samsun), mechanical stimulation or damage caused GABA to accumulate within 2 min from mean levels of 14 to 37 and 169 nmol g\(^{-1}\) fresh weight (FW), respectively. In the transgenic tobacco strain CaMVGAD27c overexpressing Petunia GAD, the same treatments caused GABA to accumulate from 12 to 59 and 279 nmol g\(^{-1}\) FW, respectively. In the transgenic tobacco strain CaMVGAD\(\Delta C11\) overexpressing Petunia GAD lacking an autoinhibitory domain, mechanical stimulation or damage caused GABA to accumulate from 180 to 309 and 630 nmol g\(^{-1}\) FW, respectively. Ambulatory activity by tobacco budworm (TBW) larvae (Heliothis virescens) on leaves of CaMVGAD27c tobacco caused GABA to accumulate from 28 to 80 nmol g\(^{-1}\) FW within 5 min. Ambulatory and leaf-rolling activity by oblique banded leaf roller (OBLR) larvae (Choristoneura rosaceana cv Harris) on wt soybean leaves (Glycine max cv Harovinton) caused GABA to accumulate from 60 to 1123 nmol g\(^{-1}\) FW within 20 min. Increased GABA levels in leaf tissue were shown to affect phytophagous preference in TBW larvae presented with wt and transgenic tobacco leaves. When presented with leaves of Samsun wt and
CaMVGAD27c plants, TBW larvae consumed more *wt* leaf tissue (640 ± 501 S.D. mm²) than transgenic leaf tissue (278 ± 338 S.D. mm²) nine times out of ten. When presented with leaves of Samsun *wt* and CaMVGADΔC11 plants, TBW larvae consumed more transgenic leaf tissue (1219 ± 1009 S.D. mm²) than *wt* leaf tissue (28 ± 31 S.D. mm²) ten times out of ten.

These results indicate that: (1) ambulatory activity of insect larvae on leaves results in increased GABA levels, (2) transgenic tobacco leaves with increased capacity for GABA synthesis deter feeding, and (3) transgenic tobacco leaves with constitutively higher GABA levels stimulate feeding.
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6. Introduction

Gamma-aminobutyric acid (GABA) is a ubiquitous, four-carbon, non-protein amino acid that has an amino group on the gamma-carbon rather than the alpha-carbon. It exists in both bound (Larher et al., 1983) and unbound forms and is highly soluble in water. GABA is flexible in aqueous solution and may adopt a cyclic configuration similar to that of proline (Christensen et al., 1994). At physiological pH it is zwitterionic, carrying both positive and negative charges (pK values of 4.03 and 10.56) (Shelp et al., 1999).

Unlike other naturally occurring non-protein amino acids that are restricted to a single species or group of closely related species, GABA is very widely distributed. Its natural occurrence was first demonstrated in potato tubers (Steward et al., 1949), and subsequently demonstrated in virtually all prokaryotic and eukaryotic organisms (Satya Narayan and Nair, 1990). GABA constitutes a significant component of the free amino acid pool and exists in virtually all plant tissues as well as root nodules of certain legumes and in xylem and phloem sap (Satya Narayan and Nair, 1990).

Resting levels of unbound GABA in plant tissue typically range from 0.03 to 2.00 μmol g⁻¹ fresh weight (FW) (Shelp et al., 1999), while bound forms may account for up to 6.6% of the total dry weight of root nodules in legumes (Larher et al., 1983). However, whether bound GABA is a significant fraction of the total GABA pool in other tissues is not clear.

GABA accumulates significantly in plant tissue in response to various biotic and abiotic stresses such as touch, cold shock, anoxia and acidosis. Two mechanisms involving increases in the cytosolic levels of H⁺ and Ca²⁺ have been implicated in stress-induced GABA accumulation. Possible roles for GABA accumulation include pH regulation, Krebs cycle bypass, nitrogen storage,
regulation of plant development and plant resistance (reviewed in Bown and Shelp, 1997; Shelp et al., 1999).

The present study investigates the hypothesis that GABA accumulation in leaf tissue in response to insect activity may function as a plant resistance mechanism against phytophagous activity of insects. GABA accumulates in plant tissue in response to the mechanical stress of touch (Knight et al., 1991). This study will determine whether the stress of insect ambulatory activity also causes GABA accumulation in leaf tissue. In insects GABA functions as an inhibitory neurotransmitter (Sattelle, 1990). This study will determine whether elevated levels of GABA ingested by an insect feeding on a plant deter the insect from feeding.
7. Literature Review

7.1 GABA Metabolism

GABA is an intermediate in the conversion of L-glutamate to succinate by a set of reactions collectively known as the GABA shunt. The three steps in the shunt are: (1) the irreversible alpha-decarboxylation of L-glutamate by L-glutamate decarboxylase (GAD) (EC 4.1.1.15) to form GABA; (2) the reversible transamination of GABA by GABA transaminase (GABA-T) (EC 2.6.1.19) with either pyruvate or 2-oxoglutarate as an amino acceptor to form succinic semialdehyde (SSA) and either alanine or glutamate; and (3) the irreversible oxidation of SSA by succinic semialdehyde dehydrogenase (SSADH) (EC 1.2.1.16) to yield the Kreb’s cycle intermediate succinate (Bown and Shelp, 1989).

\[
\text{L-glutamate} + \text{H}^+ \xrightarrow{\text{GAD}} \text{gamma-aminobutyric acid} + \text{CO}_2 \quad (1)
\]

\[
\text{gamma-aminobutyric acid} + \text{pyruvate} \xrightarrow{\text{GABA-T}} \text{succinic semialdehyde} + \text{alanine} \quad (2)
\]

\[
\text{NAD}^+ + \text{succinic semialdehyde} \xrightarrow{\text{SSADH}} \text{succinate} + \text{NADH} + \text{H}^+ \quad (3)
\]

GAD is a cytosolic enzyme (Breitkreuz and Shelp, 1995) which is specific for L-glutamate, possesses an autoinhibitory calmodulin-binding domain (Snedden et al., 1996) and has a sharp pH optimum of 5.8 (Snedden et al., 1996). GABA-T and SSADH are mitochondrial enzymes (Breitkreuz and Shelp, 1995) with pH optima of 8 to 10 and 9, respectively. Purified pyruvate-specific, tobacco-mitochondrial GABA-T exhibits Michaelis constants \(K_m\) of 1.2 mM and 0.24 mM for GABA and pyruvate, respectively (Van Cauwenberghe and Shelp, 1999). Partially purified SSADH exhibits apparent \(K_m\) of 166-460 \(\mu\)M and 5-15 \(\mu\)M for NAD\(^+\) and SSA, respectively (Shelp
et al., 1999). GABA is synthesized in the cytosol by GAD and must be transported to the mitochondria for subsequent conversion to succinate by GABA-T and SSADH. The transport of GABA into plant mitochondria has not been biochemically characterized, however, the Arabidopsis H*-coupled transport proteins ProT2 and AAP3, which transport both GABA and proline, have been identified through expression in the plasma membrane of yeast (Breitkreuz et al., 1999).

7.2 GABA Accumulation in Response to Stress

Rapid and large GABA accumulation has been reported in response to many biotic and abiotic stresses. GABA accumulation was characterized in soybean (Glycine max) leaves in response to temperature shock, darkness and mechanical manipulation (Wallace et al., 1984). Within 5 min of stress application GABA levels increased 20- to 40-fold from resting levels of 0.05 μmol/gFW to elevated levels of 1 to 2 μmol/gFW. This increase in GABA levels was accompanied by a corresponding decrease in L-glutamate concentration. Additionally, in vitro GAD activity at 30 °C in soybean leaf homogenates exceeded GABA-pyruvate transaminase activity approximately 30-fold. After separation by centrifugation, GAD was found to be associated with the soluble fraction and GABA-pyruvate transaminase with the chloroplast-enriched and mitochondrial fractions. The rapidity of stress-induced GABA synthesis indicates that increased GAD activity does not result from de novo synthesis of new enzyme. Since glutamate (Mills and Joy, 1980) and GAD are both localized in the cytosol and since it was unlikely that the substrate is sequestered away from the enzyme, it was proposed that stress-induced GABA accumulation was probably due to activation of GAD by an effector molecule such as Ca²⁺. However, at this time there were no data indicating
the involvement of \( \text{Ca}^{2+} \) in GAD activation.

Rapid GABA accumulation has also been reported in response to other plant stresses including mechanical damage, cytosolic acidification, hypoxia and water stress. Several studies have documented stress-induced GABA accumulation by directly measuring GABA levels. Mechanical damage to soybean leaflets caused increased GABA levels in leaf tissue of 10- to 25-fold within 1 to 4 min (Ramputh and Bown, 1996). Treatments resulting in cytosolic acidification of mechanically isolated *Apsaragus sprengeri* mesophyll cells caused intracellular and extracellular GABA to increase up to 914% (Crawford *et al.*, 1994). Rice seedlings exposed to anoxic conditions for 24 hr accumulated GABA from control levels of approximately 0.5 \( \mu \text{mol g}^{-1} \text{ FW} \) up to 3.5 and 6.3 \( \mu \text{mol g}^{-1} \text{ FW} \) for roots and shoots, respectively (Aurisano *et al.*, 1995). Other studies have reported stress-induced increases in the rate of GABA synthesis. In protoplasts from carrot cell suspension a decrease in pH of 0.2 units resulted in an increase in GAD activity of 80% (from 85 to 140 nkat/g protein within 2 h (Carroll *et al.*, 1994). In suspension cultured tomato cells rates of GABA synthesis increased from 0.8 \( \mu \text{mol h}^{-1} \text{ g}^{-1} \text{ FW} \) for unadapted cells to 2.4 \( \mu \text{mol h}^{-1} \text{ g}^{-1} \text{ FW} \) for water stress-adapted cells (Rhodes *et al.*, 1986).

### 7.3 Mechanisms of GABA Accumulation

Two well-documented mechanisms for GABA accumulation involve the activation of GAD. The first mechanism involves reduced cytosolic pH while the second involves increased levels of cytosolic \( \text{Ca}^{2+} \).

**REDUCED CYTOSOLIC PH:** In the absence of calcium and calmodulin, *in vitro* GAD activity
displays a pH optimum of 5.5, with activity increased approximately 40-fold over activity at pH 7 (Snedden et al., 1996). It was demonstrated that the addition of L-[U-14C]glutamate to a suspension of mechanically isolated Asparagus sprengeri mesophyll cells resulted in the alkalinization of the cell medium, the uptake of L-[U-14C]glutamate by the cells and the efflux of L-[U-14C]4-aminobutyrate (Snedden et al., 1992). The alkalinization of the medium and the influx of L-[U-14C]glutamate was taken as evidence for the influx of H+ into the cells via a H+/L-glutamate symport. The potential resulting acidification of the cytosol was taken with the efflux of L-[U-14C]4-aminobutyrate as evidence for the activation of in vivo GAD by reduced cytosolic pH.

This mechanism was later supported by in vivo results demonstrating that reduced cytosolic pH precedes GABA accumulation (Crawford et al., 1994). The cytosol of mechanically isolated Asparagus sprengeri mesophyll cells was acidified using either hypoxia, H+/L-glutamic acid symport, or the weak permeant acid butyrate. Resulting increases in total extracellular and intracellular GABA from 2.16 ± 0.39 to 4.73 ± 0.20 nmol/10⁶ cells, 3.44 ± 0.48 to 34.83 ± 3.65 nmol/10⁶ cells and 3.86 ± 0.56 to 20.36 ± 2.16 nmol/10⁶ cells for the respective treatments were reported.

**increased cytosolic Ca²⁺:** It is unlikely that all types of stress-induced GABA accumulation result from the activation of GAD by H⁺ since not all of the GABA-inducing stresses are known to reduce cytosolic pH (Bown and Shelp, 1997; Shelp et al., 1999). The stresses of touch and cold shock, however, have been reported to cause increases in cytosolic Ca²⁺ (Knight et al., 1991) and GABA (Wallace et al., 1984).

Ca²⁺/calmodulin has been shown to stimulate in vitro GAD activity at neutral pH, but not at
null
pH values below 6.5 (Snedden et al., 1996). This stimulation was blocked by calmodulin antagonists. Conversely, in the absence of calcium/calmodulin GAD activity was fully stimulated by a monoclonal antibody specific for the calmodulin-binding C-terminal region of the enzyme.

Cold shock has been reported to cause in vivo increases in cytosolic Ca\(^{2+}\) (less than 2 sec) and GABA synthesis (less than 1 min), but without an accompanying increase in H\(^{+}\) (Cholewa et al., 1997). Additionally, a calcium ionophore was used to increase calcium levels and GABA synthesis in the absence of cold shock, while a calmodulin antagonist blocked cold shock-stimulated GABA synthesis but not H\(^{+}\)-stimulated GABA synthesis.

Thus, in vivo and in vitro data indicate that Ca\(^{2+}\)- and H\(^{+}\)-mediated GABA synthesis occur independently of each other, that either is sufficient to stimulate GABA synthesis, and that Ca\(^{2+}\)/calmodulin activates GAD by binding to an autoinhibitory region on the enzyme.

### 7.4 Roles of GABA Accumulation

GABA synthesis may play several roles including cytosolic pH regulation, an alternate route for glutamate C entry into the Kreb’s Cycle, nitrogen storage, regulation of plant development, compatible osmolite in stress situations and plant defense (Shelp et al., 1999).

Anaerobiosis which may result in vivo due to flooding causes cytosolic acidification (Aurisano et al., 1995). Since GABA synthesis is stimulated by (pH optimum of 5.8) and consumes H\(^{+}\), it was proposed that H\(^{+}\)-induced GABA accumulation may serve to ameliorate cytosolic acidification (Snedden et al., 1992). Evidence to support this proposal was provided by Crawford et al. (1994) who demonstrated that in vivo GABA levels increased from approximately 3.0 to 7.5
nmol / 10^6 cells within 45 s of a 0.6 decrease in pH in response to treatment with the weak acid butyrate. Butyric acid uptake during the same timespan was 10 nmol / 10^6 cells, so H^+-consuming GABA synthesis accounted for approximately 45% of the acid load. Carroll et al. (1994) also provided evidence to support the proposal by demonstrating that in carrot protoplast suspension a decrease in cytosolic pH of 0.2 units resulted in an increase in GAD activity (from 85 to 140 nkat/g protein) within 2 h. GAD activity declined to less than 50 nkat/g protein as the pH recovered. Thus, two studies show that in vivo GABA synthesis in response to reduced cytosolic pH may function to ameliorate cytosolic acidification.

Glutamate C entry into the Krebs Cycle may occur at 2-oxoglutarate or at succinate via the GABA Shunt. Stresses such as hypoxia cause a reduction in the [NAD]/[NADH] ratio through reduction in respiration. A low [NAD]/[NADH] ratio can result in reduced NAD-dependent succinate synthesis and increased succinic semialdehyde and GABA accumulation (Satya Narayan and Nair, 1990). Upon the release of stress the accumulated GABA would provide an immediate substrate for the entry of carbon into the Krebs Cycle at succinate via succinic semialdehyde (Wallace et al., 1984). However, the metabolism of glutamate to succinate via the GABA Shunt is less energetically favourable (1 NADH) than via the Krebs Cycle (1 NADH, 1 ATP) (Bown and Shelp, 1997; Shelp et al., 1999).

GABA synthesis increases under conditions in which glutamine synthesis cannot occur or there is increased proteolysis or reduced protein synthesis. This led to the proposal that GABA acts as a temporary nitrogen storage compound during stress conditions (Satya Narayan and Nair, 1990). It has been reported that the disappearance of nitrogen from glutamate and GABA within 3 h of
initiation of protein synthesis accounts for approximately 50% of the nitrogen required for protein synthesis, and that the metabolism of glutamate into protein via the GABA Shunt is equivalent to the direct incorporation of glutamate into protein (Shelp et al., 1999). Evidence has also been reported that glutamate and GABA accumulate during seed protein storage and mobilization in developing soybean cotyledons allowing the recycling of arginine-derived N and C (Micallef and Shelp, 1989). Significant amounts of free and bound GABA in root nodules of some legumes suggests a similar nitrogen storage role following N2 fixation in root nodules (Narayan and Nair, 1990). These data suggest that GABA synthesis and metabolism may function in three different contexts: as a storage mechanism for nitrogen during stress conditions, as an intermediate in normal protein synthesis during seed development and as a product of nitrogen fixation in legume root nodules.

GABA may play a role in regulation of plant development. It is found in all plant tissues and its mRNA levels are developmentally regulated (Chen et al., 1994). It has been reported that transgenic tobacco plants expressing a mutant GAD lacking the autoinhibitory calmodulin-binding domain exhibit increased GABA levels and decreased glutamate levels in both leaf and stem, and exhibit decreased stem elongation (Baum et al., 1996). Additionally, independent studies have shown that mechanical manipulation causes increased cytosolic Ca^{2+} (Knight et al., 1991), increased GABA (Ramputh and Bown, 1996) and decreased stem elongation (Jones and Mitchell, 1989). This growth inhibition was reduced by calcium chelators and calmodulin antagonists (Ford et al., 1996). GABA synthesis and efflux of GABA from Asparagus sprengeri mesophyll cells has also been documented (Chung et al., 1992). These data suggest that stress-induced GABA accumulation and
efflux may represent an intercellular signaling process that regulates plant growth and development (Bown and Shelp, 1997). However, as yet there is no evidence for plasma membrane-localized GABA receptors homologous to the plasma membrane-localized GABA receptors in animal cells.

An osmolyte is a cellular solute which is used in some significant way to maintain or adjust cell osmotic pressure and volume. This is especially true for compounds which accumulate to high millimolar concentrations in response to osmotic shrinkage. Osmolytes which accumulate to high concentrations in the cytosol without interfering with the macromolecular functions of the cell are said to be compatible. A solution to the abiotic stresses of dessication, freezing, osmotic- and water-stress is the regulation of cell volume via the transport of inorganic ions and the accumulation of organic osmolytes. Elevated concentrations of inorganic ions may be tolerated during short-term exposure to stress. However, elevated levels of organic osmolytes are essential during long-term adaptation to stress during which sustained elevations in inorganic ion concentrations would be detrimental (Yancey, 1994). GABA, proline and glycine betaine are zwitterionic at neutral pH, are highly soluble in water and can accumulate to low mM concentrations with no apparent toxic effects to the cell (Shelp et al., 1999). Cytosolic GABA concentrations of 25 to 200 mM have been shown to stabilize and protect isolated thylakoids from freezing damage in the presence of salt (Heber et al., 1971). The transporter ProT2 from tomato pollen is activated by water stress, and transports GABA, proline and glycine betaine which are all stress-related compounds and putative compatible osmolytes (Schwacke et al., 1999). These results suggest a role for GABA in cryoprotection, however it remains unclear whether GABA may function as a compatible osmolyte or as a metabolite in the synthesis other known osmolytes such as proline (Shelp et al., 1999)
The potential role of GABA synthesis as a plant resistance mechanism is dealt with later in this literature review.

7.5 Role of GABA in Animals

GABA is the most widely distributed inhibitory neurotransmitter in vertebrate and invertebrate nervous system (Sivilotti and Nistri, 1991; Sattelle, 1990). In vertebrates three different classes of GABA receptor have been identified: GABA$_A$, GABA$_B$, and GABA$_C$ receptors.

GABA$_A$ receptors are bicuculline-sensitive, modulated by benzodiazepines and barbiturates and are linked to a Cl$^-$ channel (Kerr and Ong, 1992). GABA$_B$ receptors are baclofen-sensitive and regulate Ca$^{2+}$ and K$^+$ channels via G-proteins (Slaughter, 1995). GABA$_C$ receptors are bicuculline- and baclofen-insensitive, but are activated by GABA analogues (Feigenspan et al., 1993). They are linked to a Cl$^-$ channel, but are not modulated by benzodiazepines or barbiturates (Woodward et al., 1991)

GABA is a highly flexible molecule which may attain several low-energy conformations thus allowing it to bind to structurally dissimilar GABA receptor sites. Conformationally restricted GABA analogues may bind to a GABA receptor site if they resemble a GABA conformation which binds to the site. Curiously, the conformationally restricted GABA analogues cis-4-aminocrotonic acid (CACA) and trans-4-aminocrotonic acid (TACA) both activate GABA$_C$ receptors despite their dissimilar conformations. CACA has a cyclic conformation, while TACA has an elongated conformation (Chebib and Johnston, 1999).

Vertebrate GABA$_A$ and GABA$_C$ receptors are ionotropic. That is they belong to a large
superfamily of ligand-gated ion channels that mediate fast synaptic inhibition; specifically they are GABA-gated Cl⁻ channels. GABAₐ and GABAₐ receptors are comprised of five identical subunits which arrange together to form an ion channel and which each possess an intracellular loop believed to be a target for protein kinases (MacDonald and Olsen, 1994). GABAₐ and GABAₐ receptors are functionally different with respect to several electrophysiological criteria. GABAₐ receptors are approximately 10-fold more sensitive to GABA (EC₅₀ of 1 to 4 μM for GABAₐ, 5 to 100 μM for GABAₐ), have a lower Cl⁻ conductance (7 pS for GABAₐ, 27 to 30 pS for GABAₐ) and a longer open time (150 to 200 ms for GABAₐ, 25 to 30 ms for GABAₐ) than GABAₐ receptors (Bormann, 2000). Also they have very different pharmacological profiles based on the action of agonists and antagonists, but very little is known about modulators of GABAₐ receptors. (Chebib and Johnston, 1999).

GABAₐ receptors are metabotropic; they couple to G proteins, modulate the release of neurotransmitters such as GABA, L-glutamate, noradrenaline and dopamine and mediate slow, prolonged inhibitory signals. They modulate synaptic transmission by activating the second messenger systems phospholipase C and adenylate cyclase and by inhibiting adenylyl cyclase. These second messengers lead to the activation of plasma membrane located K⁺ and Ca²⁺ ion channels via G-coupled proteins (Kaupmann et al., 1997).

Oscillating [Ca²⁺]ᵢ pulses from 100 to 200 nM up to 350 to 600 nM occur without external stimulus in melanotrophs of the Xenopus pituitary gland. Although the inhibitory effect of GABA in suppressing these [Ca²⁺]ᵢ pulses is well documented the receptor mechanisms involved are less clear. It has been shown that selective antagonists of GABAₐ and GABAₐ receptors fail to
effectively antagonize the inhibitory response to GABA when the antagonists were given alone (Shibuya et al., 1997). However, when the antagonists were administered together the inhibitory response was completely suppressed. The data provides evidence that both GABA\textsubscript{A} and GABA\textsubscript{B} receptors participate in the GABA-induced suppression of \([Ca^{2+}]_i\) pulses in a dual-receptor mechanism. The physiological role of such a dual-receptor mechanism remains unclear, however, it is suggested that it may assure inhibition in conditions where G-protein-coupled inhibitory mechanisms do not function or where ligand-gated Cl\textsuperscript{-} channels densensitize.

Insect GABA receptors have not been as well characterized as vertebrate GABA receptors. Insect GABA receptors most closely resembled vertebrate GABA\textsubscript{A} receptors because they are linked to a Cl\textsuperscript{-} channel and are modulated by benzodiazepines and barbiturates (Taylor et al., 1993). However, insect GABA receptors are generally insensitive to bicuculline and have been found to be activated by CACA and TACA providing evidence that they may resemble vertebrate GABA\textsubscript{C} receptors (Taylor et al., 1993). These pharmacological results lead to ambiguity regarding the physiological nature of insect GABA receptors. Recently, a complete pharmacological profile of insect GABA receptors was performed utilizing agonists, chloride channel blockers, competitive GABA\textsubscript{A} receptor antagonists, competitive GABA\textsubscript{C} receptor antagonists and modulators (Aydar and Beadle, 1999). The study found that insect GABA receptors display a novel pharmacology and do not conform to either vertebrate GABA\textsubscript{A} or GABA\textsubscript{C} receptors.

Many commercial pesticides such as polychlorocycloalkanes, bicyclophosphorous esters and bicycloorthobenzoates are agonists or antagonists of GABA-gated Cl\textsuperscript{-} channels (Casida, 1993). Neuromuscular junctions may be exposed to these pesticides either \textit{via} absorption through the cuticle
or via ingestion and passage through the haemolymph (Irving et al., 1976).
7.6 Role of GABA in Insect Gustation

Gustation is the predominant method by which animals select food. This is especially true in insects in which feeding specialization on a single host plant or group of host plants is common. Although visual, olfactory and physical cues play roles in host seeking and acceptance, contact chemoperception of non-volatile nutrients and secondary chemicals normally determine feeding. Acceptance of a host plant requires sufficient chemoperception of phagostimulants relative to a threshold level of antifeedants which may not be exceeded (Mullin et al., 1994).

GABA, glycine and related aminobutyric acids are known phagostimulants in four herbivorous insect orders: Orthoptera, Homoptera, Coleoptera and Lepidoptera. GABA and glycine have been shown to elicit immediate firing responses from individual taste cells of western corn rootworm larvae (Diabrotica) implicating GABA receptors in insect gustation (Mullin et al., 1994). Furthermore, antagonists of GABA<sub>A</sub> receptors such as beta-hydrastine and bicuculline have been shown to be strongly antifeedant (ED<sub>50</sub> of 122 and 166 μM, respectively) in a squash disk bioassay with Diabrotica. ED<sub>50</sub> represents the concentration of an antifeedant which results in a 50% reduction in feeding as compared with the control. Antagonists of GABA<sub>B</sub> receptors such as 2-hydroxysaclofen did not have antifeedant effects. This suggests that an insect GABA receptor similar to the vertebrate GABA<sub>A</sub> receptor is involved in taste chemoperception and gustation in insects (Mullin et al., 1994).
7.7 Putative Role of GABA Accumulation as a Plant Resistance Response to Phytophagous Insects

Mechanical stimulation causes increases in cytosolic Ca\(^{2+}\) (Knight et al., 1991), through activation of a mechanosensitive calcium-selective cation channel (MCaC) (Zimmermann et al., 1997). Mechanical damage will cause increases in cytosolic H\(^{+}\) and Ca\(^{2+}\) levels through disruption of cellular compartmentation and release of vacuolar H\(^{+}\) and Ca\(^{2+}\). Thus it is reasonable to attribute rapid GABA synthesis in response to mechanical stimulation or damage (Wallace et al., 1984) to H\(^{+}\)-and Ca\(^{2+}\)-stimulated increases in GAD activity (Ramputh and Bown, 1996).

Similarly, mechanical stimulation or damage to leaf tissue by insect ambulatory or phytophagous activity respectively will presumable cause increases in cytosolic H\(^{+}\) and Ca\(^{2+}\) levels. This will activate GAD and cause rapid GABA synthesis. The inhibitory effect of elevated GABA levels on the growth and development of insect larvae was studied (Ramputh and Bown, 1996). Mechanical damage to soybean leaf tissue similar to that resulting from phytophagous insect activity caused a 10- to 25-fold increase in leaf GABA levels to 2.15 ± 0.11 μmol GABA g\(^{-1}\) FW within 1 to 4 min. Comparable GABA levels of 1.6 to 11.6 μmol g\(^{-1}\) FW in artificial diets resulted in increased mortality, increased time to pupation and decreased mass of oblique-banded leaf roller larvae reared on the diets. Commercial insecticides are thought to disrupt normal neuromuscular activity and contain active ingredients that are agonists or antagonists of the GABA-gated Cl\(^{-}\) channel (Casida, 1993). It is possible therefore that ingested dietary GABA may have a similar disruptive effect (Bown and Shelp, 1997).
7.8 Hypothesis

The present study investigates the hypothesis that increased GABA levels in leaf tissue in response to insect activity may function as a plant resistance mechanism against phytophagous insect activity. In order to validate this hypothesis two criteria must be satisfied; it must be shown that:

(1) insect activity causes increased GABA levels in leaf tissue, and

(2) increased GABA levels in leaf tissue deter insects from feeding.

To test the first criterion leaf tissue was subjected to insect larval ambulatory activity, and the resulting effects on leaf GABA levels was determined. To test the second criterion the phytophagous preference of insect larvae for leaves containing high or low GABA levels in a two-choice preference experiment was determined.
8. Materials and Methods

8.1 Materials

8.1.1 Chemicals

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<td>Tobacco Budworm Larval Diet</td>
<td>Products Inc.</td>
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<td>Tobacco Budworm Diet Dry Mix</td>
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8.1.2 Biological Material

8.1.2.1 Soybean Plants

Soybean seeds of the strain *Glycine max* cv Harovinton were obtained from Agriculture and Agri-Food Canada (Harrow, Ontario).

8.1.2.2 Tobacco Plants

Seeds for the following tobacco strains of *Nicotiana tabacum* were obtained from Dr. Barry Shelp at Guelph University (Guelph, Ontario): Samsun *wt*, Delgold *wt*, Samsun CaMVGAD27a, Samsun CaMVGAD27c, Samsun CaMVGAD33a and Samsun CaMVGAD33b. The wild type strains are commercially available agricultural strains, while the transgenic strains express a foreign gene coding for the enzyme petunia glutamate decarboxylase (GAD) which was cloned, sequenced and analysed by Dr. Hillel Fromm at The Weizmann Institute for Science (Rehovot, Israel) (Baum *et al.* 1993). The following tobacco strains grown from tissue culture were obtained from Dr. Barry Shelp at University of Guelph (Guelph, Ontario): Samsun *wt*, Samsun CaMVGADΔC11 and Samsun CaMVGADΔC29. These transgenics contain a petunia GAD gene coding for a GAD enzyme lacking the autoinhibitory domain.

The transgenic strains CaMVGAD and CaMVGADΔC were transformed using the *Agrobacterium tumefaciens* binary vector system which involves a large tumour inducing (*Ti*) plasmid and a small shuttle vector. The *Ti* plasmid contains virulence genes used to facilitate the transport of the shuttle vector from *Agrobacterium* into the target plant cell. The shuttle vector contains left- and right-border tDNA, the cauliflower mosaic virus (CaMV) promoter, the Kanamycin
resistance (Kan') selectable marker gene and the gene of interest, GAD or GADΔC.

Data indicating expression of the transgenic Kan' marker gene and morphological differences in CaMVGAD were provided with the plant seeds. Approximately 100 seeds from Samsun wt, CaMVGAD27c and CaMVGAD33b were distributed on Murashige-Skooge medium agarose plates containing 50 μg mL⁻¹ kanamycin sulfate which is known to block chlorophyll synthesis. Approximately 100 Samsun wt seeds were also distributed on a plate not containing kanamycin. The frequency of germination for all seed sets was greater than 95%. Samsun wt seedlings which sprouted on plates not containing kanamycin grew and synthesized chlorophyll, while Samsun wt seedlings which sprouted on plates containing kanamycin stopped growing and bleached (Fig. 1). All CaMVGAD27c and CaMVGAD33b seedlings which sprouted grew and synthesized chlorophyll, indicating expression of the Kan' marker gene. Additionally, the flowers of mature Samsun wt and CaMVGAD27c plants are morphologically different (Fig. 2). While the flowers of Samsun wt plants appear normal, the flowers of CaMVGAD27c plants exhibit partial or full petaloidy in which some or all of the stamens develop as petals. The kanamycin resistance assay data suggest that expression of transgenic Petunia GAD occurs in the CaMVGAD strains and that it may result in morphological differences between the transgenic and wt strains (Mike McLean, personal communication, University of Guelph).
Figure 1. Kanamycin Resistance Assay. Approximately 100 seeds from Samsun wt, CaMV/GAD27c and CaMV/GAD33b were distributed on MS medium agarose plates either containing or not containing 50 μg mL⁻¹ kanamycin sulfate: Samsun wt + kanamycin (a), CaMV/GAD27c + kanamycin (b), Samsun wt - kanamycin (c), CaMV/GAD33b + kanamycin (d). The rate of germination for all seed sets was greater than 95%. (Courtesy of Dr. Barry Shelp, University of Guelph)
Figure 2. Morphological differences between Samsun wt and CaM/VGAD27c tobacco strains. Mature flowers from Samsun wt plants exhibit normal morphology (a). Mature flowers from CaM/VGAD27c plants exhibit either partial (b) or full petaloidy (c). (Courtesy of Dr. Barry Shelp, University of Guelph)
Data indicating genomic integration of the transgene coding for Petunia GAD lacking an autoinhibitory domain and morphological differences in CaMVGAD were provided with the plants.  

2 to 3 µg of genomic DNA from Samsun wt, CaMVGADAC8, CaMVGADAC11, CaMVGADAC27 and CaMVGADAC29 plants was prepared, digested with BamHI and electrophoresed through 0.8% agarose gels using 0.5X TBE buffer. The DNA was hybridized in dextran sulfate using a 1.3 kbp BamHI-EcoRI probe fragment from the 3' end of Petunia GAD cDNA which was labeled to a specific activity of 2 x 109 cpm µg⁻¹. Hybridization conditions were 55° C for 48 h. The blot was washed under 0.5X SSC and 0.1% SDS at 45° C and exposed on Kodak X-OMAT film for 13 days. Band numbers and density indicate that the strains CaMVGADAC8, CaMVGADAC11, CaMVGADAC27 and CaMVGADAC29 contain 5, 3, 0 and 2 copies of transgenic Petunia GAD, respectively (Fig. 3). Additionally, mature Samsun wt plants differ morphologically from the CaMVGADAC8, CaMVGADAC11, CaMVGADAC27 and CaMVGADAC29 strains with respect to stem elongation and ramification (Fig. 4). While Samsun wt plants display normal morphology, the CaMVGADAC strains exhibit stunted growth and increased stem branching. It is likely that the expression of the transgenic Petunia GAD genes, which were identified in the Southern blot, is responsible for the morphological abnormalities exhibited by the CaMVGAD strains (Mike McLean, personal communication).
Figure 3. Southern Blot. Genomic DNA from Samsun wt, CaMVGADΔC8, CaMVGADΔC11, CaMVGADΔC27 and CaMVGADΔC29 tobacco strains was digested with BamHI, separated by electrophoresis and hybridized with a labeled 1.3 kbp BamHI-EcoRI fragment from the 3' end of Petunia GAD cDNA. The resulting blot was washed and exposed. The density of the 4.0 kbp bands indicates that the CaMVGADΔC8, CaMVGADΔC11, CaMVGADΔC27 and CaMVGADΔC29 strains contain 5, 3, 0 and 2 copies of transgenic Petunia GAD, respectively. (Courtesy of Dr. Barry Shelp, University of Guelph)
Figure 4. Morphological differences between Samsun wt and CaMV\text{GADAC} tobacco strains. The Samsun wt displays normal morphology while the CaMV\text{GADAC} strains exhibit reduced stem elongation and increased ramification: Samsun wt (a), CaMV\text{GADAC}8 (b), CaMV\text{GADAC}11 (c), CaMV\text{GADAC}27 (d), CaMV\text{GADAC}29 (e). (Courtesy of Dr. Barry Shelp, University of Guelph)
8.1.2.3 Oblique Banded Leaf Roller Larva

Egg masses of oblique banded leaf roller larvae, *Choristoneura rosaceana* cv Harris, were obtained from Dr. David Pree at Agriculture and Agri-Food Canada (Vineland, Ontario).

8.1.2.4 Tobacco Budworm Larvae

Egg masses of tobacco budworm, *Heliothis virescens*, were obtained from French Agricultural (Lamberton, MN, USA).

8.2 Methods

8.2.1 Growth of Biological Materials

8.2.1.1 Soybean Plants

Two soybean seeds were pressed by hand to a depth of 1 cm into a 4"x4" pot containing Sunshine Mix 2. Seedlings sprouted after approximately seven days, and were allowed to grow in the greenhouse under a natural light cycle for the months of March to October, 1998, at 25-35 °C, with thorough watering every other day.

8.2.1.2 Tobacco Plants

For Samsun *wt* and CaMVGAD tobacco plants, vermiculite was mixed with water and packed in seedling trays. Tobacco seeds were sown by sprinkling approximately 5-10 seeds on each
vermiculite plug. The seedling tray was covered and stored in the greenhouse. After approximately 14 days the vermiculite plugs containing tobacco seedlings were transplanted into 4"x4" pots containing Sunshine Mix 2. The seedlings were allowed to grow in the greenhouse under a natural light cycle for the months of February to November, 1998 and 1999, at 25-35 °C, with thorough watering every other day.

CamVGADAC tobacco plants derived from tissue culture were obtained from Dr. Barry Shelp (University of Guelph, Guelph, Ontario) and were allowed to grow in the greenhouse under a natural light cycle for the months of June to August, 1999, at 25-35 °C, with thorough watering every other day.

8.2.1.3 Oblique Banded Leaf Roller Larvae

Immediately upon hatching of the egg mass, oblique banded leaf roller larvae were transferred to 3.5 cm in diameter x 3.5 cm in height plastic cups (Solo Cup Co., Urbana, IL, USA) containing approximately 1 g of omnivorous leaf roller diet (Bio-Serv Inc., Frenchtown, NJ, USA). Larvae were reared at 22 °C under a 16:8 light:dark light cycle until they reached a mass of at least 140 mg.

8.2.1.4 Tobacco Budworm Larvae

Immediately upon hatching of the egg mass, tobacco budworm larvae were transferred to 3.5 cm in diameter x 3.5 cm in height plastic cups (Solo Cup Co., Urbana, IL, USA) containing approximately 1 g of artificial tobacco budworm diet (Southland Products Inc., Lake Village,
Arkansas, USA). Larvae were reared at 22 °C under a 16:8 light:dark light cycle until they reached a mass of at least 140 mg.

8.2.2 Leaf Treatments

The following leaf treatments were used to stimulate GABA synthesis in soybean and tobacco leaves prior to GABA analysis. Control leaves were not treated in any manner and were excised directly into liquid nitrogen for GABA analysis. For experiments using soybean, the control and experimental leaflets were taken from the same trifoliolate. For experiments using tobacco, the control and experimental leaves were from the same plant and were of similar size and appearance. After treatment, experimental leaves were excised directly into liquid nitrogen for GABA analysis.

8.2.2.1 Mechanical Stimulation

A tobacco leaf was rolled by hand approximately 150 times along the longitudinal axis for 1 min. The leaf was rolled to the extent that opposite edges of the leaf could touch above the upper surface of the leaf. This type of mechanical stimulation did not leave any permanent, visible signs of damage to the leaf. Upon completion of rolling, the leaf was undisturbed for a further 1 min. The leaf was then excised directly into a mortar containing liquid nitrogen for subsequent GABA analysis.

8.2.2.2 Mechanical Damage

A tobacco leaf was excised into an empty mortar and ground with a pestle for 1 min. Upon
completion of the grinding, the leaf tissue was undisturbed for a further 1 min. The leaf tissue was then transferred into a second mortar containing liquid nitrogen for subsequent GABA analysis.

8.2.2.3 OBLR Ambulatory and Leaf Rolling Activity

An oblique banded leaf roller larva weighing approximately 160 mg was placed on an attached soybean leaf and allowed to freely ambulate or roll the leaf. The insect was removed after 10 min or 20 min of ambulation. The leaf was then excised directly into liquid nitrogen for subsequent GABA analysis. Alternatively, the insect was removed after 20 min, providing that it ambulated for approximately the first 10 min and rolled the leaf for the remainder of the time (Fig. 5). The leaf was then excised directly into liquid nitrogen for subsequent GABA analysis.
Figure 5. Oblique banded leaf roller larva rolling an attached Harvinton wt soybean leaflet.
8.2.2.4 TBW Ambulatory Activity

A tobacco budworm larva weighing approximately 160 mg was placed on an attached tobacco leaf and allowed to freely ambulate for 2, 5 or 10 min before being removed. The leaf was then excised directly into liquid nitrogen for subsequent GABA analysis.

8.2.3 GABA Analysis

GABA from leaf tissue was extracted and determined using tissue samples obtained by one of the four leaf treatments described.

8.2.3.1 GABA Extraction

Liquid nitrogen in a mortar was used to freeze leaf segments, which were then ground to a fine powder using a pestle. GABA was extracted according to the procedure of Zhang and Bown (1997). An Eppendorf tube containing 400 μL methanol, which is used to inactivate GAD, was weighed. Approximately 0.1 mg of frozen tissue powder was transferred into the Eppendorf tube which was weighed again to obtain the mass of tissue powder transferred. The resulting homogenate was dried in a water bath to evaporate the supernatant methanol. 1000 μL of 70 mM LaCl₃ was added and shaken intermittently for 10 min. The Eppendorf tube was centrifuged for 5 min at 13,000 g. 800 μL of the resulting supernatant was transferred into a second Eppendorf tube containing 160 μL of 1.0 N KOH, and vigorously shaken intermittently for 3-5 min. The Eppendorf tube containing supernatant fluid and KOH was centrifuged for 5 min at 13,000 g to remove a precipitate containing yellow phenolic compounds which interfere with the GABA assay. 550 μL of the resulting
supernatant fluid was transferred to a 1 mL cuvette for subsequent GABA determination. This extraction procedure was repeated in triplicate for each sample of ground tissue.

8.2.3.2 GABA Determination

GABA was determined according to the recommended procedure from Sigma for the use of GABAse. However, this procedure was scaled down from the recommended 3.0 mL assay volume to 1.0 mL. 200 µL of 0.5 M pH 8.6 K₄P₂O₇ buffer, 150 µL of 4 mM NADP⁺, and 50 µL of 2.0 units/mL GABAse were added to a cuvette containing 550 µL of sample. The sample was mixed thoroughly and the initial absorbance measured at 340 nm. 50 µL of 20 mM α-ketoglutarate was added to the cuvette and mixed thoroughly. After 1 hr the final absorbance was measured at 340 nm. The amount of GABA in the cuvette (nmol) and the amount of GABA in the initial tissue sample (nmol/gFW) was determined using the calculated change in absorbance at 340 nm and a GABA calibration graph constructed from standard GABA samples. The calibration graph was linear for GABA levels ranging from 0 to 100 nmol of GABA.
Figure 6. GABA Calibration Graph. The change in absorbance at 340 nm is plotted against known concentrations of GABA. Each point is the mean with standard error of 6 separate GABAse analyses. A linear regression line with a non-zero intercept due to absorbance by α-ketoglutarate in the reaction mixture is shown.
8.2.4 Phytophagous Preference Experiments

All phytophagous preference experiments were conducted using a feeding chamber containing two whole tobacco leaves attached to two separate plants and one tobacco budworm larva (Fig. 7). The feeding chamber was a clear, plastic cylindrical chamber measuring 115 mm in diameter x 40 mm in height with a removable base. Two holes approximately 3 mm in diameter on opposite sides of the chamber allowed whole tobacco leaves to be placed in the chamber, one on each side, while still attached by the stem to the plant. A tobacco budworm larva was placed in the center of the chamber, equidistant from each of the leaves, and the chamber was sealed. After 20 hr the chamber was opened, the insect was removed and the leaves were excised for imaging to determine areas eaten.
Figure 7. Feeding preference chamber containing tobacco budworm larvae and attached Samsun *wt* and CaM/FGAD27c tobacco leaves.
8.2.4.1 TBW Phytophagous Preference When Supplied with Different Tobacco Strains

Two tobacco leaves were placed in the feeding chamber: one Samsun *wt* and one of Samsun CaMVGAD27c, Samsun CaMVGADΔC11 or Samsun CaMVGADΔC29. Leaves of similar age and appearance and which had dimensions of approximately 120 mm length x 80 mm width were used.

8.2.4.2 Digital Imaging of Tobacco Leaves

Upon completion of the 20 hr feeding period by the tobacco budworm larva, the leaves were excised and digitally imaged using the Analytical Imaging Station system (Imaging Research, Inc.) to determine the area of the leaf consumed by the insect. Images were captured using a COHU 4915-2001/0000 High Performance CCD Camera connected via a MuTech MV1000 Imaging Board to a Pentium class PC running Analytical Imaging Station software. Data were recorded as area of the leaf consumed in mm².

8.2.4.3 Specific Leaf Weight Determination

Leaf area consumed and biomass removed are two common measures of herbivory (Waller and Jones, 1989). However, since leaves may vary in thickness and in density these measures are not equivalent and may lead to inaccurate interpretations of data when taken alone. Leaf area and biomass are only comparable when the leaves are physically identical. Specific leaf weight (SLW) is a measure of dry weight (DW) per area of leaf tissue. Therefore, two leaves which do not vary in thickness or in density should have identical SLWs. In order to determine whether the *wt* and CaMVGAD27c tobacco leaves used in the phytophagous preference experiments were physically
similar with respect to thickness and density, their SLWs were determined. Leaves of similar age and appearance to those used in the phytophagous preference experiments were selected, and discs were cut from between their veins using a paper hole punch. The leaf discs were imaged to determine surface area and dried in a drying oven at 45 °C for 48 h. After removal from the drying the oven, the leaf discs were weighed to determined DW, and the SLW for each disc was calculated in units of g DW m².

8.2.5 Statistical Analyses

8.2.5.1 Wilcoxon Signed Rank Test

The Wilcoxon Signed Rank Test, also known as the Wilcoxon Paired-Sample Test or Wilcoxon Rank-Sum Test, is a nonparametric statistical test for paired data. The most commonly employed biostatistical procedure is the comparison of two samples to determine whether differences exist between the two populations sampled. Observations made in each sample may either be independent of each other or they may be correlated. Paired data refers to the case in which observations in the first sample are correlated to observations in the second sample. Parametric statistical tests make inferences about populations by comparing two population means, medians, variances, coefficients of variation and indices of diversity. However, these parametric tests assume a normal distribution of the data within each sample. When a normal distribution cannot be assumed, due to a small number of observations, then a transformation of the data must be performed before any statistical inferences may be made. For the Wilcoxon Signed Rank Test the data are
sorted by magnitude, and assigned ranks. Inferences are then drawn from the sum of the ranks for each sample (Zar, 1996).

The Wilcoxon Signed Rank Test was selected for testing the phytophagous preference of TBW larvae for one of two leaves offered in two-choice preference experiments. Leaf area consumed was the measure used to determine phytophagous preference. The data were considered to be paired because the total area of both leaves consumed varied greatly from one insect to another, implying that the areas of each leaf consumed were correlated by the total consumption of the insect larva. Due to the small sample size, the data were not tested for normality. Thus the Wilcoxon Signed Rank Test was selected based upon the assumptions of testing paired and nonparametric data.

The data are ranked irrespective of group, and the rank sums for each group are determined. The procedure tests the hypothesis that the distributions for the two groups are identical. If the two rank sums differ significantly from each other, then it is assumed that the distributions and central values of the two groups differ from each other (Zar, 1996).

8.2.5.2 Kruskal-Wallis One-Way Analysis of Variance

An analysis of variance (ANOVA or AOV) is a method of making inferences based on measurements of a variable from three or more samples. Parametric AOVs test the equality of means for normal samples to determine the source of variation, that is whether sample variation differs significantly from population variation. One of the underlying assumptions, however, is that the data must be normally distributed. The Kruskal-Wallis One-Way AOV is a nonparametric test which avoids the use of population parameters by making inferences based upon the rank of observations
The Kruskal-Wallis One-Way AOV was selected to test for differences in GABA levels in leaf tissue of soybean or tobacco after various treatments because in all cases multiple samples were involved and the data were not tested for normality.

The data are ranked irrespective of group, and the mean ranks for each group are determined. If the groups have similar distributions, then the mean ranks would be expected to be similar. If any of the mean ranks deviate significantly from the central mean of all the groups, then it is assumed that the distributions for the groups differ from each other. In this case it is typical to assume that the differences in distribution arise from differences in the central values of the groups. Once differences are identified a procedure known as a nonparametric multiple comparison must be employed in order to locate between which groups the difference occurs (Zar, 1996).
9. Results

9.1 GABA accumulation in attached *wt* and CaMVGAD tobacco leaves after mechanical stimulation or damage

GABA accumulation in the leaf tissue of many species has been reported in response to various physical stresses including mechanical stimulation (Wallace et al., 1984; Bown and Shelp, 1989). Before investigating the putative role of GABA accumulation as a plant resistance mechanism, it was necessary to characterize this phenomenon in *wt* tobacco and CaMVGAD tobacco strains overexpressing GAD. Tobacco leaves were either mechanically stimulated by rolling along the longitudinal axis or mechanically damaged by grinding in a mortar. After 1 min of stimulation or damage the leaf tissue was left undisturbed for a further 1 min prior to GABA analysis. Control leaves were not stimulated in any manner. In two *wt* and four transgenic strains GABA levels ranged from 9.8 to 23.1 nmol/gFW in control leaves, 37.4 to 143.5 nmol/gFW in stimulated leaves and 169.2 to 700.1 in damaged leaves (Figure 8). In each strain tested, the amounts of GABA in control, mechanically stimulated or mechanically damaged tissues were found to be significantly different. Significance was determined using the Kruskal-Wallis One-Way Analysis of Variance, rejection level $p > 0.05$. These data confirm that GABA accumulation occurs in response to mechanical stimulation or damage.
Figure 8. GABA accumulation in attached wt and CaMVGAD tobacco leaves after mechanical stimulation or damage. Control leaves were not stimulated in any manner. Experimental leaves were either mechanically stimulated or mechanically damaged for 1 min and left undisturbed for a further minute prior to GABA analysis. Data represent the mean ± standard deviation for the sample size indicated above each column. Different letters demarking columns within the same group indicate a significant difference according to the Kruskal-Wallis One-Way Analysis of Variance, rejection level p > 0.05.
The second criterion of this thesis states that increased GABA levels in leaf tissue deters phytophagous insect activity. In order to test this criterion using \( wt \) and CaMVGAD tobacco strains it was necessary to determine whether the transgenic CaMVGAD strains exhibit higher GABA levels in leaf tissue than the \( wt \) strains. The GABA accumulation data for \( wt \) and CaMVGAD strains was analyzed in order to determine whether there is an effect of GAD overexpression on GABA levels in addition to an effect of mechanical stimulation and damage. For the control and stimulated treatments, two statistically different groups were identified using the Kruskal-Wallis One-Way Analysis of Variance, rejection level \( p > 0.05 \), while for the damaged treatment three statistically different groups were identified (Fig 9). For all three treatments, there were transgenic CaMVGAD strains identified as belonging exclusively to statistical groups which exhibited the highest GABA levels. Conversely, for all treatments, there were \( wt \) strains identified as belonging exclusively to statistical groups which exhibited the lowest GABA levels. These data indicate that the transgenic CaMVGAD tobacco strains exhibit higher GABA levels in leaf tissue than the \( wt \) strains.
Figure 10. GABA accumulation in attached wt and CaMVGAD tobacco leaves after mechanical stimulation or damage. Control leaves were not stimulated in any manner. Experimental leaves were either mechanically stimulated or mechanically damaged for 1 min and left undisturbed for a further minute prior to GABA analysis. Data represent the mean ± standard deviation for the sample size indicated above each column. Different letters demarking columns within the same group indicate a significant difference according to the Kruskal-Wallis One-Way Analysis of Variance, rejection level p > 0.05.
9.2 GABA accumulation in attached *wt* and CaMVGADΔC tobacco leaves after mechanical stimulation or damage

Similarly, it was necessary to characterize GABA accumulation in response to mechanical stimulation in the *wt* and CaMVGADΔC tobacco strains. Again, tobacco leaves were either mechanically stimulated or mechanically damaged. After 1 min of stimulation or damage the leaf tissue was left undisturbed for a further 1 min prior to GABA analysis. Control leaves were not stimulated in any manner. Mean GABA levels of 6.5, 24.7 and 217.1 nmol/gFW were determined in the respective control, stimulated and damaged leaves of the *wt* strain. For the two transgenic strains, CaMVGADΔC11 and CaMVGADΔC29, mean GABA levels were 180.1 and 470.6 nmol/gFW in control leaves, 297.9 and 309.2 nmol/gFW in stimulated leaves and 630.2 and 643.0 nmol/gFW in damaged leaves, respectively (Fig. 10). The significance of these results was determined using the Kruskal-Wallis One-Way Analysis of Variance, rejection level $p > 0.05$. For the *wt* strain mean GABA levels in control and stimulated tissue were both significantly different from those found in damaged tissue, but not from each other. For the CaMVGADΔC11 strain, the mean GABA levels in control and damaged tissue were significantly different from each other, but not from those found in stimulated tissue. Finally, for the CaMVGADΔC29 strain, there was no significant difference between mean GABA levels in control, stimulated and damaged tissues.
Figure 12. GABA accumulation in attached \textit{wt} and CaMV\textit{GADdC} tobacco leaves after mechanical stimulation or damage. Control leaves were not stimulated in any manner. Experimental leaves were either mechanically stimulated or mechanically damaged for 1 min and left undisturbed for a further minute prior to GABA analysis. Data represent the mean ± standard deviation for the sample size indicated above each column. Different letters demarking columns within the same group indicate a significant difference according to the Kruskal-Wallis One-Way Analysis of Variance, rejection level $p > 0.05$. 
RESULTS

Again, in order to use the \textit{wt} and CaMVGAD\textDelta C tobacco strains in phytophagous preference experiments it was necessary to determine whether the transgenic CaMVGAD\textDelta C strains exhibit higher GABA levels in leaf tissue than the \textit{wt} strain. The GABA accumulation data for \textit{wt} and CaMVGAD\textDelta C tobacco strains was re-analyzed in order to determine whether there is an effect of overexpression of truncated GAD on GABA levels in leaf tissue in addition to an effect of mechanical stimulation or damage. For the control, stimulated and damaged treatments, three statistically different groups were identified using the Kruskal-Wallis One-Way Analysis of Variance, rejection level $p > 0.05$ (Fig. 11). For all treatments, there were CaMVGAD\textDelta C strains identified as belonging exclusively to statistical groups which exhibited the highest GABA levels. Conversely, for all treatments, the \textit{wt} strain was identified as belonging exclusively to statistical groups which exhibited the lowest GABA levels. These data indicate that the transgenic CaMVGAD\textDelta C tobacco strains exhibit higher GABA levels in leaf tissue than the \textit{wt} strain.
Figure 14. GABA accumulation in attached *wt* and *CaMVGADΔC* tobacco leaves after mechanical stimulation or damage. Control leaves were not stimulated in any manner. Experimental leaves were either mechanically stimulated or mechanically damaged for 1 min and left undisturbed for a further minute prior to GABA analysis. Data represent the mean ± standard deviation for the sample size indicated above each column. Different letters demarking columns within the same group indicate a significant difference according to the Kruskal-Wallis One-Way Analysis of Variance, rejection level $p > 0.05$. 
9.3 GABA accumulation in attached wt and CaMVGAD27c tobacco leaves after TBW ambulatory activity

The first criterion of this thesis states that GABA accumulates in leaf tissue as a response to ambulatory and phytophagous insect activity. In order to test this criterion, wt and CaMVGAD27c tobacco leaves were tested for GABA accumulation after ambulatory stimulation by a tobacco budworm larva. A TBW larva was placed on an attached tobacco leaf and allowed to freely ambulate for 2, 5 or 10 min before being removed. The leaf was then excised directly into liquid nitrogen for subsequent GABA analysis. Control leaves were not treated in any manner, but were excised into liquid nitrogen prior to GABA analysis. The data for both tobacco strains were divided into two categories: large leaves (area > 2500 mm\(^2\)) and small leaves (area < 1500 mm\(^2\)). Statistical significance of the results was determined using the Kruskal-Wallis One-Way Analysis of Variance, rejection level \(p > 0.05\).

For the large wt leaves, mean GABA levels ranged from 24.3 to 28.7 nmol/gFW for the control and all three treatment times. For the large transgenic leaves, mean GABA levels ranged from 32.5 to 50.7 nmol/gFW for the control and all three treatment times. For the control and transgenic strains there were no significant differences in GABA accumulation for control or stimulated leaves.

However, for the small wt leaves, mean GABA levels ranged from 12.2 to 66.9 nmol/gFW for the control and all three treatment times. For the small transgenic leaves, mean GABA levels ranged from 28.4 to 80.2 nmol/gFW for the control and all three treatment times. For the wt strain, 5 min and 10 min treatments were significantly different from the control, while the 2 min treatment was not significantly different from any of the other treatments or control. For the CaMVGAD27c
strain, the pattern of significance was the same with the exception that the 2 min treatment was different from the 5 min treatment.

The data indicate that TBW ambulatory activity does not cause GABA accumulation on a whole leaf basis in leaves with surface area greater than 2500 mm\(^2\), but that it does cause GABA accumulation in leaves with surface area less than 1500 mm\(^2\). This may result from a localized GABA response to insect stimulation being masked in a large leaf by unstimulated tissue.
Figure 16. GABA accumulation in attached wt and CaMVGAD27c tobacco leaves after TBW ambulatory activity. Control leaves were not stimulated in any manner. Experimental leaves were stimulated by TBW ambulatory activity for 2, 5 or 10 min. All leaves were immediately excised for subsequent GABA analysis. Data represent the mean ± standard deviation for the sample size indicated above each column. Different letters demarking columns within the same group indicate a significant difference according to the Kruskal-Wallis One-Way Analysis of Variance, rejection level p > 0.05.
9.4 GABA accumulation in attached *wt* Harovinton soybean leaflets after physical stimulation by OBLR larva

GABA accumulates in tobacco leaves in response to ambulatory activity by TBW larvae (Fig. 12). However, in order determine whether this phenomenon is specific to the TBW-tobacco system or whether it may be a typical response to insect activity in plants, it was necessary to test another system. An oblique banded leaf roller larva was placed on an attached soybean leaflet and allowed to freely ambulate or roll the leaflet. The insect was removed after 10 or 20 min of ambulation or after 20 min providing that it ambulated for approximately the first 10 min and rolled the leaflet for the remainder of the time. After removal of the insect from the leaflet, the leaflet was excised directly into liquid nitrogen for subsequent GABA analysis. GABA levels in the leaflet tissue were found to be 59.6 ± 18.1, 668.9 ± 74.9, 884.4 ± 89.4 and 1122.8 ± 122.5 nmol / gFW for the control, 10 min ambulation, 20 min ambulation and 20 min ambulation and leaf rolling data, respectively (Fig. 13). All results were found to be significantly different from each other as determined by the Kruskal-Wallis One-Way Analysis of Variance, rejection level p > 0.05, with the exception of the data for 20 min of ambulatory activity which was only different from the control. Thus the data indicate that GABA accumulation in response to insect activity is not specific to the TBW-tobacco system.
Figure 18. GABA accumulation in attached wt Harovinton soybean leaflets after physical stimulation by OBLR larva. Control leaflets were not stimulated in any manner. Experimental leaflets were stimulated by 10 min ambulatory activity by an OBLR larva, 20 min ambulatory activity or 10 min ambulatory activity followed immediately by 10 min leaf-rolling activity. All leaflets were immediately excised directly into liquid nitrogen, and ground into a fine powder for subsequent GABA analysis. Data represent the mean ± standard deviation for the sample size indicated above each column. Different letters demarking columns indicate a significant difference according to the Kruskal-Wallis One-Way Analysis of Variance, rejection level p > 0.05.
9.5 Phytophagous Preference of TBW Larvae Feeding on wt or CaMVGAD Tobacco

The second criterion of this thesis states that GABA accumulation in leaf tissue deters phytophagous insect activity. In order to test this criterion the phytophagous preference of TBW larvae for either wt tobacco with normal capacity for GABA accumulation or CaMVGAD27c tobacco with increased capacity for GABA accumulation was tested (Table 1). A tobacco budworm larva was placed in the center of a feeding chamber containing two whole tobacco leaves (one control leaf and one experimental leaf) attached to two separate plants, and the chamber was sealed. The control leaf was from Samsun wt, and the experimental leaf was from CaMVGAD27c. After 20 h the chamber was opened, the insect was removed and the leaves were excised for imaging to determine the areas consumed.

Ten trials were performed (Fig 14, Table 1). The mean leaf areas consumed by the larvae were 639.9 ± 500.9 S.D. mm² for the Samsun wt leaf and 278.3 ± 338.0 S.D. mm² for the CaMVGAD27c leaf (Table 1). In nine out of ten trials the wt leaf was preferred. However, the total area of leaf tissue consumed by the TBW larvae in each pair-wise trial varied considerably. Consequently, the standard deviations are large. The result was found to be significant using the Wilcoxon Signed Rank Test which considers that data pair-wise (1-tailed, p = 0.0186). The data indicate that a phytophagous preference for Samsun wt leaves over CaMVGAD27c leaves exists in tobacco budworm larvae.
Table 1: Phytophagous preference of TBW larvae for *wt* tobacco over CaMVGAD27c tobacco.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Samsun wt</th>
<th>Samsun CaMVGAD27c</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>815.2</td>
<td>85.4</td>
</tr>
<tr>
<td>B</td>
<td>885.4</td>
<td>576.9</td>
</tr>
<tr>
<td>C</td>
<td>649.3</td>
<td>1074.7</td>
</tr>
<tr>
<td>D</td>
<td>395.6</td>
<td>0.0</td>
</tr>
<tr>
<td>E</td>
<td>350.4</td>
<td>13.6</td>
</tr>
<tr>
<td>F</td>
<td>152.1</td>
<td>97.9</td>
</tr>
<tr>
<td>G</td>
<td>1746.5</td>
<td>378.9</td>
</tr>
<tr>
<td>H</td>
<td>980.1</td>
<td>288.7</td>
</tr>
<tr>
<td>I</td>
<td>411.0</td>
<td>266.1</td>
</tr>
<tr>
<td>J</td>
<td>12.9</td>
<td>0.7</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>6398.5</strong></td>
<td><strong>2782.8</strong></td>
</tr>
<tr>
<td><strong>Mean</strong></td>
<td><strong>639.9</strong></td>
<td><strong>278.3</strong></td>
</tr>
<tr>
<td><strong>Standard Deviation</strong></td>
<td><strong>500.9</strong></td>
<td><strong>338</strong></td>
</tr>
</tbody>
</table>

Two whole, attached tobacco leaves, one *wt* and one CaMVGAD27c, were placed in a feeding chamber, one TBW larva was added and the chamber was closed. After 20 h the chamber was opened, the larva was removed and the leaves were excised for subsequent imaging to determine the areas of each leaf eaten. Total *wt* and CaMVGAD27c leaf areas consumed by TBW larvae were determined to be significantly different using the Wilcoxon Signed Rank Test (1-tailed), \( p = 0.0186 \).
Figure 20. Phytophagous preference of TBW larvae for wt tobacco over CaMV GAD27c tobacco. These digital images were captured immediately after removal of the insect larva from the feeding preference chamber, and were used to determine the leaf areas consumed which appear in Table 1.
9.6 Phytophagous Preference of TBW Larvae Feeding on wt or CaMVGADAC Tobacco

To further investigate the second criterion of this thesis, which states that GABA accumulation deters phytophagous insect activity, the phytophagous preference of TBW larvae for either wt tobacco with normal GABA levels or CaMVGADAC transgenic strains with constitutively higher GABA levels was tested (Tables 2-3). A tobacco budworm larva was placed in the center of a feeding chamber containing two whole tobacco leaves (one control leaf and one experimental) attached to two separate plants, and the chamber was sealed. The control leaf was from Samsun wt, and the experimental leaf was from either CaMVGADAC11 or CaMVGADAC29. After 20 h the chamber was opened, the insect was removed and the leaves were excised for imaging to determine the areas consumed.

For preference experiments involving Samsun wt and CaMVGADAC11 strains, ten trials were performed (Fig 15, Table 2). The mean leaf areas consumed by the larvae were 27.6 ± 30.6 S.D. mm² for the Samsun wt leaf and 1219.2 ± 1008.5 S.D. mm² for the CaMVGADAC11 leaf (Table 2). In ten out of ten trials the transgenic leaf was preferred. Again standard deviations are large due to considerable variations in the total area of leaf tissue consumed by the TBW larvae in each pair-wise trial. The result was found to be significant using the Wilcoxon Signed Rank Test (1-tailed, p = 0.0010). The data indicate that a phytophagous preference for CaMVGADAC11 leaves over Samsun wt leaves exists in tobacco budworm larvae.

For preference experiments involving Samsun wt and CaMVGADAC29 strains, eight trials were performed (Fig 16, Table 3). The mean leaf areas consumed by the larvae were 158.0 ± 307.7
S.D. mm$^2$ for the Samsun wt leaf and 614.9 ± 505.1 s.d. mm$^2$ for the CaMVGADΔC29 leaf (Table 3). In seven out of eight trials the transgenic leaf was preferred. The result was not found to be significant using the Wilcoxon Signed Rank Test (1-tailed, p = 0.0547). The data do not indicate a phytophagous preference for Samsun wt leaves or CaMVGADΔC29 leaves in tobacco budworm larvae.
Table 2: Phytophagous preference of TBW larvae for CaMVGADAC11 tobacco over wt tobacco.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Samsun wt</th>
<th>Samsun CaMVGADAC11</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>92.7</td>
<td>3051.3</td>
</tr>
<tr>
<td>B</td>
<td>45.5</td>
<td>873.7</td>
</tr>
<tr>
<td>C</td>
<td>23.1</td>
<td>1483</td>
</tr>
<tr>
<td>D</td>
<td>45.6</td>
<td>481.2</td>
</tr>
<tr>
<td>E</td>
<td>49.1</td>
<td>1287.2</td>
</tr>
<tr>
<td>F</td>
<td>0</td>
<td>42.1</td>
</tr>
<tr>
<td>G</td>
<td>0</td>
<td>298.3</td>
</tr>
<tr>
<td>H</td>
<td>0.6</td>
<td>1323.7</td>
</tr>
<tr>
<td>I</td>
<td>19.6</td>
<td>2770.6</td>
</tr>
<tr>
<td>J</td>
<td>0</td>
<td>581.2</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>276.2</strong></td>
<td><strong>12192.3</strong></td>
</tr>
<tr>
<td><strong>Mean</strong></td>
<td><strong>27.6</strong></td>
<td><strong>1219.2</strong></td>
</tr>
<tr>
<td><strong>Standard Deviation</strong></td>
<td><strong>30.6</strong></td>
<td><strong>1008.5</strong></td>
</tr>
</tbody>
</table>

Two whole, attached tobacco leaves, one wt and one CaMVGADAC11, were placed in a feeding chamber, one TBW larva was added and the chamber was closed. After 20 h the chamber was opened, the larva was removed and the leaves were excised for subsequent imaging to determine the areas of each leaf eaten. Total wt and CaMVGADAC11 leaf areas consumed by TBW larvae were determined to be significantly different using the Wilcoxon Signed Rank Test (1-tailed), $p = 0.0010$. 
<table>
<thead>
<tr>
<th>Code</th>
<th>Overall</th>
<th>Contact</th>
</tr>
</thead>
<tbody>
<tr>
<td>1200</td>
<td>4.00</td>
<td>3.00</td>
</tr>
<tr>
<td>1190</td>
<td>4.70</td>
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<td>1170</td>
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<td>1160</td>
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<tr>
<td>1150</td>
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<td>1140</td>
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<td>1130</td>
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</tr>
<tr>
<td>1100</td>
<td>4.70</td>
<td></td>
</tr>
</tbody>
</table>

[Additional text not visible in the image]
Figure 22. Phytophagous preference of TBW larvae for CaMVGADΔC11 tobacco over wt tobacco. These digital images were captured immediately after removal of the insect larva from the feeding preference chamber, and were used to determine the leaf areas consumed which appear in Table 2. The image for A-CaMVGADΔC11 is not included because the corresponding data file became corrupted.
Table 3: Phytophagous preference of TBW larvae for CaMVGADAC29 tobacco over wt tobacco.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Samsun wt</th>
<th>Samsun CaMVGADAC29</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>11.4</td>
<td>468.5</td>
</tr>
<tr>
<td>B</td>
<td>17.2</td>
<td>314.6</td>
</tr>
<tr>
<td>C</td>
<td>0</td>
<td>1524.7</td>
</tr>
<tr>
<td>D</td>
<td>1.4</td>
<td>1186.3</td>
</tr>
<tr>
<td>E</td>
<td>206.4</td>
<td>444</td>
</tr>
<tr>
<td>F</td>
<td>5.6</td>
<td>725.5</td>
</tr>
<tr>
<td>G</td>
<td>896.5</td>
<td>82.3</td>
</tr>
<tr>
<td>H</td>
<td>125.8</td>
<td>173.6</td>
</tr>
<tr>
<td>Total</td>
<td>1264.4</td>
<td>4919.6</td>
</tr>
<tr>
<td>Mean</td>
<td>158</td>
<td>614.9</td>
</tr>
<tr>
<td>Standard Deviation</td>
<td>307.7</td>
<td>505.1</td>
</tr>
</tbody>
</table>

Two whole, attached tobacco leaves, one wt and one CaMVGADAC29, were placed in a feeding chamber, one TBW larva was added and the chamber was closed. After 20 h the chamber was opened, the larva was removed and the leaves were excised for subsequent imaging to determine the areas of each leaf eaten. Total wt and CaMVGADAC29 leaf areas consumed by TBW larvae were determined to not be significantly different using the Wilcoxon Signed Rank Test (1-tailed), p = 0.0547.
Figure 24. Phytophagous preference of TBW larvae for CaMVGAΔC29 tobacco over wt tobacco. These digital images were captured immediately after removal of the insect larva from the feeding preference chamber, and were used to determine the leaf areas consumed which appear in Table 3.
10. Discussion

10.1 Chemical Ecology

The term *chemical ecology* was coined by Sondheimer and Simeone (1970) to describe the field of biology which investigates the chemical basis for ecological interactions between organisms. For example, Dethier (1970) uses the chemical mechanism of feeding deterrents and feeding stimulants in plant tissue interacting with insect taste receptors to explain the evolution of feeding diversity in the host-plant associations of herbivorous insects. Before discussing the putative role of GABA as an ecological chemical in plant resistance to phytophagous insect activity, it is necessary to define some potentially ambiguous terms.

10.1.1 Plant Resistance and Defense

*Resistance* is the degree to which a plant avoids damage by herbivores (Rausher, 1992). It is a relative quantity rather than absolute and can only be quantified with reference to a standard. Experimentally, resistance is commonly measured as the inverse of an amount or proportion of plant tissue that is damaged or eaten by herbivores. Resistance is determined both by genetic and environmental factors. Genetic factors control plant traits that deter feeding or oviposition behaviour in herbivores. Environmental factors influence both the expression of plant resistance traits and the abundance and behaviour of herbivores. A *resistance trait* is any inherited plant character that reduces the amount of damage a plant suffers under a specific environmental regime.

*Defense* is resistance that has evolved or is maintained in a plant population because of
selection exerted by herbivores or other natural enemies (Rausher, 1992). In order to qualify as a defensive trait a resistance trait must satisfy three criteria: (1) there must be genetic variation for the resistance trait; (2) the genetic variation for the trait must be subject to selection; and (3) the selection on the trait must be imposed by herbivores. Thus the term defense implies an evolutionary origin or role, while the term resistance does not.

*Tolerance* is the ability of a plant to experience damage without a resulting reduction in fitness (Rausher, 1992). As with resistance it is a relative quantity, and it is influenced both by genetic and environmental factors. It should be noted that when a plant tolerates herbivore damage with no resulting reduction in fitness then selection may not be exerted on genetically variable resistance traits.

Chemical resistance may be either quantitative or qualitative (Feeny, 1970). *Quantitative* resistance chemicals reduce digestibility or palatability in a concentration dependent manner and often function against many different herbivores. Because they are more effective at higher concentrations in plant tissue they are costly to produce. *Qualitative* resistance chemicals have specific molecular actions and are effective at low concentrations, usually against specific species or groups of species of herbivores. They are less costly for the plant to produce. Quantitative and qualitative resistance traits are commonly present in apparent and unapparent plants, respectively. *Apparent* plants are those plants that because of their size or longevity are bound to be found by herbivores. They generally employ quantitative resistance chemicals which function against a broad range of herbivores. *Unapparent* plants are plants which may escape from herbivores in time or space. They may avoid predation by appearing when herbivores are not present or by being small
in size or discreetly located. They employ qualitative resistance chemicals which function against specific herbivores. Quantitative resistance theory predicts that the content of resistance chemicals in apparent plants should be inversely correlated with the degree of herbivory.

10.1.2 Classification of Semiochemicals

_Semiochemicals_ are chemicals that mediate interactions between organisms (Schoonhoven _et al._, 1998). With respect to insect-plant interactions they may be classified based on the responses they elicit from insects into five categories including attractant, repellant, arrestant, deterrent and stimulant (Dethier, 1960). An *attractant* is a chemical which causes insects to make oriented movements towards its source. Conversely, a *repellant* is a chemical which causes insects to make oriented movements away from its source. An *arrestant* is a chemical which slows the linear progression of an insect either by reducing the speed of locomotion or by increasing the turning rate. A *deterrent* is a chemical which inhibits feeding or oviposition behaviour when present in a place where insects would, in its absence, feed or oviposit. Two subclassifications of stimulant have been defined. A *locomotor stimulant* is a chemical which causes insects to disperse from a region more rapidly than if the area did not contain the chemical. A *feeding, mating or ovipositional stimulant* is a chemical which elicits feeding, mating or oviposition in insects. The term feeding stimulant is synonymous with phagostimulant. These terms may not be necessarily exclusive of each other. For example, a semiochemical may play a role as both an arrestant and a phagostimulant under the same set of conditions (Chamberlain _et al._, 2000).
10.1.3 GABA as a Semiochemical

The present study investigates the hypothesis that increased GABA levels in leaf tissue may function as a plant resistance mechanism against phytophagous insect activity. In this role GABA would function as a feeding deterrent. In order to support the hypothesis, it is necessary to demonstrate that increased GABA levels inhibit feeding behaviour when present in leaves that insects would normally, under normal GABA levels, feed on. Two testable predictions of the hypothesis are: (1) insect activity causes increased GABA levels in leaf tissue, and (2) increased GABA levels in leaf tissue deter feeding. In this capacity GABA would presumably function as a concentration-dependent, quantitative resistance chemical.

10.2 GABA Synthesis and Accumulation

The first criterion of the present study states that insect activity causes increased GABA levels in leaf tissue. Insect activity includes activities such as walking on, rolling and feeding on leaves. The effect of these activities on leaf tissue GABA levels was investigated, as was the effect of simulated activities such as mechanical rolling of a leaf and crushing of a leaf to simulate leaf rolling and feeding, respectively.

10.2.1 Mechanisms of GABA Synthesis

It has been demonstrated that touch stress causes increases in cytosolic Ca\(^{2+}\) (Knight et al., 1991) and GABA (Wallace et al., 1984), and that Ca\(^{2+}\)/calmodulin stimulates \textit{in vitro} GAD activity by binding to an autoinhibitory region on the enzyme (Snedden et al., 1996). Also, the stimulation
of mechanosensitive ion channel activity in response to the mechanical stress of suction has been documented (Zimmerman et al., 1997). In the present study mechanical stimulation of *wt* and CaMVGAD tobacco leaves caused significant accumulation of GABA in leaf tissue (Fig. 8). The mechanical stress of rolling the leaf for 1 min would activate plasma membrane mechanosensitive ion channels allowing Ca$^{2+}$ to enter into the cytosol from the apoplast. Increased cytosolic Ca$^{2+}$ would activate GAD and cause GABA synthesis in both *wt* and CaMVGAD strains. However, it was demonstrated that CaMVGAD strains accumulated approximately 2.5 times more GABA in response to mechanical stimulation than did *wt* strains (Fig. 9). This increased capacity for GABA synthesis in response to mechanical stress in the CaMVGAD transgenics is likely due to overexpression of the Petunia GAD enzyme (M. MacLean, personal communication).

*In vitro* GAD activity displays a pH optimum of 5.5 in the absence of Ca$^{2+}$/calmodulin (Snedden et al., 1996), and *in vivo* cytosolic acidification has been shown to stimulate intracellular and extracellular GABA accumulation (Crawford et al., 1994). Mechanical damage to *wt* and CaMVGAD tobacco leaves caused significant accumulation of GABA in leaf tissue (Fig. 8). The leaf tissue was crushed with a mortar and pestle for 1 min which would disrupt cellular compartmentation by damaging both plasma and vacuolar membranes. This would release apoplastic Ca$^{2+}$ and vacuolar Ca$^{2+}$ and H$^{+}$ stores into the cytosol. Thus GABA accumulation in both *wt* and CaMVGAD strains would likely be due to Ca$^{2+}$- and H$^{+}$-activation of GAD. However, it was demonstrated that CaMVGAD strains accumulated more GABA in response to mechanical damage than did *wt* strains (Fig. 9). Again, this increased capacity for GABA synthesis in the CaMVGAD transgenics is likely due to overexpression of the Petunia GAD enzyme.
DISCUSSION

It is expected that GABA synthesis and accumulation will differ in CaMVGADAC tobacco strains since they overexpress Petunia GAD lacking an Ca\textsuperscript{2+}/calmodulin-binding autoinhibitory domain. The resting level of GABA in these transgenics is expected to be higher than in the \textit{wt} strain because a GAD enzyme lacking an autoinhibitory domain will not require a Ca\textsuperscript{2+}/calmodulin signal to activate catalytic activity. Therefore, it should synthesize GABA even under low [Ca\textsuperscript{2+}] conditions. This prediction is true since resting levels in CaMVGADAC\textit{C11} and CaMVGADAC\textit{C29} were 180.1 and 470.6 nmol gFW\textsuperscript{-1} as opposed to resting levels of 6.5 nmol gFW\textsuperscript{-1} in Samsun \textit{wt} (Fig. 10).

Little or no accumulation is expected in the CaMVGADAC transgenics in response to mechanical stimulation which causes increases in cytosolic Ca\textsuperscript{2+}. Since the GAD in the transgenics lacks the Ca\textsuperscript{2+}/calmodulin-binding autoinhibitory domain the enzyme is already activated, and should not be sensitive to increases in cytosolic Ca\textsuperscript{2+}. Mechanical stimulation did not cause a significant increase in GABA levels in CaMVGADAC\textit{C11} or CaMVGADAC\textit{C29} plants when compared to control values for the same strains (Fig. 11). It is possible that some GABA accumulation will occur in the transgenics due to the presence of the native, tobacco Ca\textsuperscript{2+}/calmodulin-sensitive GAD. However, the prediction that little or no accumulation is expected in the transgenics in response to mechanical stimulation was confirmed since neither of these GABA levels were significantly different from their respective controls.

Finally, GABA accumulation is expected in the CaMVGADAC transgenics in response to mechanical damage since H\textsuperscript{+} activation of GAD is independent of the Ca\textsuperscript{2+}/calmodulin-binding domain. This prediction is true for the CaMVGADAC\textit{C11} strain in which mechanical damage caused
a significant 3-fold increase in GABA levels (Fig. 11). However, the prediction is not true for the CaMVGADAC29 strain in which mechanical damage did not cause a significant increase in GABA levels (Fig. 11).

The predictions that CaMVGADAC tobacco strains overexpressing Petunia GAD lacking a Ca$^{2+}$/calmodulin-binding autoinhibitory domain would exhibit higher resting levels of GABA, little or no accumulation of GABA in response to mechanical stimulation, and accumulation of GABA in response to mechanical damage were found to be true with one exception. The CaMVGADAC29 strain failed to exhibit significant increases in GABA in response to mechanical damage. Although the reason for this unexpected result is not clear, it could be attributed to either a small sample size, a difference in copy number of the transgene or low cytosolic L-glu levels due to conversion to GABA. It is possible that the sample size was insufficiently large to discriminate between differences in control GABA levels and GABA levels induced by mechanical damage. Additionally, it is possible that the GABA increase in response to mechanical damage was smaller in the CaMVGADAC29 strain than in the CaMVGADAC11 strain because the copy numbers of the Petunia CaMVGADAC gene are 5 and 2, respectively (M. MacLean, personal communication). The remaining predicted results which hold true indicate that the CaMVGADAC tobacco strains are activated independent of Ca$^{2+}$/calmodulin, and that they may be further activated by H$^+$. 

10.2.2 GABA Synthesis in Response to Insect Activity

In the present study, the effect of insect activities on GABA levels in leaf tissue was examined in two different systems. The effect of TBW larva ambulatory activity on GABA levels
in tobacco leaves was studied. Additionally, the effect of OBLR larva ambulatory and leaf-rolling activity on GABA levels in soybean leaflets was examined.

The mechanical stimulation which may result from various insect activities was simulated by hand prior to examining the insect-plant systems. Resting GABA levels in leaf tissue of Samsun wt and CaMVGAD27c plants were 13.5 and 11.9 nmol gFW⁻¹, respectively (Fig. 8). Leaves from both strains were mechanically stimulated by rolling the along the longitudinal axis by hand for 1 min. After stimulation GABA levels had increased significantly to 37.4 and 58.7 nmol gFW⁻¹ for the wt and transgenic respectively (Fig. 8).

The effect of TBW ambulatory activity on GABA levels in Samsun wt and CaMVGAD27c leaves was examined. Initially, TBW larvae were allowed to ambulate on mature tobacco leaves for 2, 5 or 10 min, after which time the leaf was excised and the GABA content determined. The leaves were of similar size and shape and were all greater than 2500 mm² in area. GABA levels after ambulation did not differ significantly 24.3 to 28.7 and 32.5 to 50.7 nmol gFW⁻¹ for the wt and transgenic strains respectively (Fig. 12). Neither strain exhibited a significant increase in leaf GABA levels after ambulation by the insect. The experiment was repeated using immature tobacco leaves in place of mature leaves. The small tobacco leaves were of similar size and shape and were all less than 1500 mm² in area. For the Samsun wt strain GABA levels increased significantly from 12.2 nmol gFW⁻¹ for the control to 41.6, 56.0 and 66.9 nmol gFW⁻¹ for the 2, 5 and 10 min ambulation times respectively (Fig. 12). For the CaMVGAD27c strain GABA levels increased from 28.4 nmol gFW⁻¹ for the control to 50.4, 80.2 and 70.9 nmol gFW⁻¹ for the 2, 5 and 10 min ambulation times respectively (Fig. 12). For both strains the increase in leaf GABA levels was significant for the 5
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and 10 min ambulation times. The fact that TBW larva ambulation caused significant increases in leaf GABA levels in the small immature leaves but not in the large mature leaves may be due to a dilution effect. For the small, immature leaves, the TBW larvae ambulated over nearly the entire leaf surface. Presumably this ambulation induced increases in GABA over a large portion of the leaf, which were detectable after excising and analyzing the entire leaf. However, for the large, mature leaves, the TBW larvae only ambulated over portions of the leaf surface. It is likely that this ambulation induced localized increases in GABA over a smaller fraction of the leaf. Theses increases were not detected due to dilution by unstimulated tissue.

The effect of OBLR ambulatory and leaf-rolling activities on GABA levels in Harovinton wt soybean leaflets was examined (Fig. 13). An OBLR larva was placed on a soybean leaflet and allowed to ambulate or roll the leaf. The larva was removed after 10 or 20 min of ambulation or after 20 min providing that it ambulated for approximately the first 10 min and rolled the leaf for the remaining time. GABA levels in the leaflets were found to be 59.6, 668.9, 884.4 and 1122.8 nmol gFW$^{-1}$ for the control, 10 min ambulation, 20 min ambulation and 20 min ambulation and leaf rolling activities, respectively. All of the treatments were significantly different from the control, and the 10 min ambulation treatment was significantly different from the 20 min ambulation and leaf rolling treatment.

GABA accumulation in leaf tissue in response to mechanical stimulation was first reported by Wallace et al. (1984). He suggested that induced accumulation of GABA in response to insect feeding activity was likely, and that because of GABA’s role as a neurotransmitter in insects, it was likely that GABA accumulation in leaf tissue may affect insect feeding behaviour. The present study
provides the first documentation that GABA accumulates in response to insect activity. GABA accumulation was shown to be induced in tobacco by TBW ambulatory activity and in soybean by OBLR ambulatory and leaf-rolling activity. These activities involve mechanical stimulation of leaf tissue which would presumably activate plasma membrane mechanosensitive ion channels. Thus the major mechanism for a GABA accumulation response would be Ca^{2+}/calmodulin-mediated increases in GAD activity. These findings support the first criterion of the hypothesis that insect activity causes increased GABA levels in leaf tissue.

However, it is difficult to document GABA accumulation in response to insect phytophagous activity. This activity involves mechanical damage to leaf tissue and would release apoplastic Ca^{2+} and vacuolar Ca^{2+} and H^{+} stores. Crushing of soybean leaf tissue has been shown to result in a sap pH of 5.4 (Ramputh and Bown, 1996), likely due to the release of vacuolar H^{+}. Since GAD has an acidic pH optimum of 5.8 at which Ca^{2+}/calmodulin is not stimulatory, it is likely that a GABA accumulation response would involve only H^{+}-mediated increases in GAD activity. Attempting to analyze GABA increases in a partially consumed leaf is not feasible because the response appears to be highly localized, as suggested by the data for TBW ambulation on small and large tobacco leaves (Fig. 12). GABA accumulation in areas of the leaf which have been chewed would be diluted by the remaining, unstimulated tissue upon analyzing the entire remaining leaf. Additionally, a significant portion of the leaf in which GABA accumulation has occurred would have been consumed by the insect. It may be possible to analyze the gut contents, haemolymph or frass of the insect for GABA. However, only a positive result would be meaningful. If a negative result were obtained, it could be interpreted as meaning that GABA did not accumulate or that GABA did
accumulate but was absorbed and possibly metabolized by the insect.

10.3 GABA and Insect Gustation

The second criterion of the present study states that increased GABA levels in leaf tissue deter insects from feeding. The effect of elevated GABA levels in leaf tissue on host plant selection was investigated through the use of two-choice feeding preference assays.

10.3.1 Host-Plant Selection

Host-plant selection behaviour in insects is a behaviour which follows a predictable sequence of events that is organized into two main phases: the searching phase and the evaluation phase (Schoonhoven et al., 1998). Both of these phases involve several sequential events. During these events, the insect exhibits behavioural reactions appropriate to the various relevant stimuli. These stimuli include visual and physical plant cues as well as chemical cues including semiochemicals such as attractants, repellants, arrestants, stimulants and deterrents. A typical 7-step host-plant selection sequence is described:

1. The *searching phase* begins with the event of dispersal. During *dispersal* an insect has no physical contact with a plant and either rests or moves about randomly.

2. The insect perceives plant-derived optical or olfactory cues.

3. The insect responds to these cues in such a way that the physical distance between the insect and the plant decreases.

4. The searching phase ends with the event of finding. *Finding* occurs when the plant is
physically contacted by the insect.

5. The evaluation phase begins when the insect examines the plant surface by contact-testing, or palpation.

6. Further examination involves damage to the plant and release of tissue contents by test-biting, probing or puncturing with an ovipositor.

7. The evaluation phase ends with the events of either acceptance or rejection. Acceptance occurs if either feeding continues or one or more eggs are laid. Rejection occurs if the insect departs without accepting the plant.

During each event the insect may react positively by moving forward through the sequence to the next event or it may react negatively by returning to a previous event. These reactions are mediated by the plant-derived cues which the insect perceives. Progressing through the sequence of events, the insect is subjected to an increasing number and intensity of plant cues (Schoonhoven et al., 1998). It is important to note that the reactions of the insect to these cues, including the crucial decision of whether to accept or reject a plant, are modified by the insect's physiological status (satiety, sexual maturity, egg maturation) (Barton Brown, 1993) and by information about previous experiences stored in the insect's memory (Dethier, 1982). Thus under different conditions, the same stimuli may result in different reactions by the insect.

Acceptance is a straightforward term which describes sustained feeding or ovipositioning behaviour by an insect on a single plant. When more than one plant is available to be accepted, other terms may be used to describe host-plant selection. Selection is a term which describes the act of choosing between alternatives and which requires differential sensory perception of alternative food
plants. If evaluation of potential host plants is made sequentially, then a short-term memory is required to make comparisons over time. In any case where more than one potential host plant has been evaluated prior to final acceptance the term selection is appropriate. Selection describes one acceptance event on a single plant. When in a dual or multiple choice assay an insect consistently selects one of the potential host plant species or strains more often than the others the use of the term preference is appropriate. The corollary of this in nature or under field conditions is when the degree of feeding or oviposition on a potential host species is higher than would be predicted by its relative abundance. Finally, the term recognition refers to the neural process of recognizing a host plant. Sensory input of plant-derived cues is compared to an internal image of an acceptable host plant which is stored in the central nervous system (CNS). If the incoming sensory information sufficiently matches the stored image, then the plant is recognized as a host plant.

10.3.2 GABA as a Phagostimulant

The second criterion of this study, that increased GABA levels in leaf tissue deter insects from feeding, involves the event of acceptance and specifically implies selection and preference. The potential role of GABA as a semiochemical mediating this event is necessarily that of either phagostimulant or feeding deterrent. Two-choice feeding preference experiments involving TBW larvae and strains of tobacco exhibiting either various constitutive leaf tissue GABA levels or different potentials for stress-induced GABA production were employed to test this criterion.

Preference assays were performed by placing one TBW larva in a feeding chamber with one whole, attached Samsun wt leaf and one of either a whole, attached CaMVGADΔC11 or
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CaMVGADAC29 leaf. The larva was allowed to feed for 20 h, after which it was removed and the area consumed on each leaf was determined (Tables 2-3). Samsun wt tobacco was previously shown to accumulate leaf tissue GABA (from resting levels of 6.5 nmol gFW\(^{-1}\)) to 217.1 nmol gFW\(^{-1}\) in response to mechanical damage which would simulate insect phytophagous activity. Similarly, CaMVGADAC11 and CaMVGADAC29 strains accumulated leaf tissue GABA (from resting levels of 180.1 and 470.6 nmol gFW\(^{-1}\)) to 630.2 and 643.0 nmol gFW\(^{-1}\), respectively, in response to the same mechanical damage. In trials between Samsun wt and CaMVGADAC11 tobacco TBW larvae consumed mean leaf areas of 27.6 and 1219.2 mm\(^2\), respectively. This preference for the CaMVGADAC11 strain was found to be significant using the Wilcoxon Signed Rank Test (1-tailed, p = 0.0010). In trials between Samsun wt and CaMVGADAC29 tobacco TBW larvae consumed mean leaf areas of 158.0 and 614.9 mm\(^2\), respectively. This preference for the CaMVGADAC29 strain was found to be nearly significant (p = 0.0547). Together these results indicate that TBW larvae preferred feeding on CaMVGADAC tobacco strains that exhibited higher leaf tissue GABA levels as opposed to Samsun wt tobacco, and imply that GABA plays a role as a phagostimulant in host plant selection.

This finding is in agreement with literature demonstrating that GABA stimulates feeding and evokes taste cell responses in several species of herbivorous insects including the Colorado potato beetle *Leptinotarsa decemlineata* (Thorsteinson, 1960), grasshopper *Cannula pellucida* (Thorsteinson, 1960), aphid *Acyrthosiphon pisum* (Srivastava *et al.*, 1983) and western corn rootworm beetle *Diabrotica virgifera virgifera* (Mullin *et al.*, 1992). Insect gustatory sensilla, which are located on the mouthparts and tarsi, are the organs which mediate chemoperception of taste
chemicals such phagostimulants and feeding deterrents. Each sensillum contains several sensory neurons, or taste cells, which are separated from the outside environment by a single cuticular pore at the tip of the sensillum. Since there is no membrane or barrier, dendritic receptors on taste cells are directly exposed to all external chemicals which enter the pore. Morphologically, the external chemosphere of the insect is analogous to an extended synaptic cleft of the taste cell (Mullin et al., 1994). GABA has been shown to be equally effective at stimulating taste cells in insect gustatory sensilla as some other known phagostimulants. In the Colorado potato beetle, GABA, L-alanine and sucrose stimulate the same taste cell in a maxillary sensillum with an ED$_{50}$ (concentration at half-maximal spike frequency) of 1.69, 1.65 and 1 mM, respectively (Mitchell and Harrison, 1984). In the western corn rootworm, GABA and glycine also stimulate the same taste cell in a maxillary sensillum with an ED$_{50}$ of 100 µM (Mullin et al., 1994). This literature suggests that increased GABA levels in the CaMVGADΔC tobacco strains may play a phagostimulatory role in TBW gustation by stimulating taste cell resulting in preference of these strains over the Samsun wt strain. While this result may agree with current literature, it fails to support the second criteria of this hypothesis, that increased GABA levels in leaf tissue deters insects from feeding.

10.3.3 GABA as a Feeding Deterrent

In addition to the two-choice preference experiments involving Samsun wt and CaMVGADΔC tobacco strains, the second criterion of the present study was also addressed using preference experiments between Samsun wt and CaMVGAD strains. A TBW larva was placed in a feeding preference chamber with one whole, attached Samsun wt leaf and one whole, attached
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CaMVGAD27c leaf. The larva was allowed to feed for 20 h, after which it was removed and the area consumed on each leaf was determined (Table 1). Samsun wt tobacco was previously shown to accumulate leaf tissue GABA (from resting levels of 13.5 nmol gFW\(^{-1}\)) to 169.2 nmol gFW\(^{-1}\) in response to mechanical damage which would simulate insect phytophagous activity. Similarly, CaMVGAD27c accumulated leaf tissue GABA (from resting levels of 11.6 nmol gFW\(^{-1}\)) to 279.4 nmol gFW\(^{-1}\) in response to the same mechanical damage. TBW larvae consumed mean leaf areas of 639.9 and 278.3 mm\(^2\) for the Samsun wt and CaMVGAD27c strains, respectively. This preference for the Samsun wt strain was found to be significant using the Wilcoxon Signed Rank Test (1-tailed, \(p = 0.0186\)). This result indicates that TBW larvae preferred feeding on the Samsun wt leaf tissue which exhibited lower GABA levels than the CaMVGAD27c leaf tissue. It also implies that GABA plays a role as a feeding deterrent in host plant selection which supports the second criterion of the present study that increased GABA levels in leaf tissue deters insects from feeding.

However, it is possible that the potential role of GABA as a feeding deterrent is not gustatory in nature, but rather due to neurophysiological toxicity. GABA is a known inhibitory neurotransmitter which transiently hyperpolarizes the neural membrane by activating a Cl\(^-\) channel and allowing Cl\(^-\) influx (Sattelle, 1990). Several synthetic organic insecticides, including cyclodienes and polychlorocycloalkanes (PCCAs), are known to function by acting on GABA-gated Cl\(^-\) channels. PCCAs such as lindane, hexachlorocyclohexane, toxaphene, aldrin, dieldrin and endosulfan act on the GABAergic system by blocking GABA-gated Cl\(^-\) channels (Lawrence and Casida, 1984). Picrotoxinin (PTX), the active agent in the insecticide picrotoxin, acts as a GABA antagonist by binding a noncompetitive blocker (NCB) site on the mammalian GABA\(\alpha_{\text{A}}\) receptor.
(Leeb-Lundberg and Olsen, 1980). In all there are at least three known sites of insecticide action on the GABAergic system with the NCB site being affected by 2 distinct modes of action (Casida, 1993).

In order for ingested GABA to function as a neurological toxin it is necessary for GABA to be absorbed from the gut into the haemolymph where it may affect the insect nervous system. The midgut of lepidopteran larvae is an epithelium composed primarily of columnar and goblet cells. Columnar cells are absorptive cells which possess a well-developed brush border and a K⁺-dependent amino acid symport (Hanozet et al., 1980). Goblet cells excrete K⁺ and are responsible for maintaining the K⁺ electrochemical gradient which drives amino acid uptake (Harvey and Nedergaard, 1964). Neutral amino acid absorption involves transport from the midgut into a columnar cell across the mucosal border, mixing in the cytoplasmic pool, and subsequent excretion from the cell into the surrounding tissue across the basal membrane (Hanozet et al., 1980). This transport is sodium-independent, mediated by a K⁺-dependent symport, and has been demonstrated in isolated brush border membrane vesicles from several lepidopteran species including silkworm *Philosamia cynthia* (Hanozet et al., 1980) and tobacco hornworm *Manduca sexta* (Hennigan et al., 1993).

In order for GABA in the haemolymph to act as a neurophysiological toxin it is necessary that it can access the nervous system. This is possible because insect GABA receptors, especially those on muscles, are exposed to the haemolymph (Lunt, 1991). The neuromuscular junctions of many insect species, including the blowfly *Lucilia sericata* and *Calliphora vicina*, are not covered by glial cells. Therefore, there is no cellular barrier between the synapse and the haemolymph. This
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anatomical observation is supported by behavioural studies in which L-glutamate injected directly into the haemolymph caused reversible paralysis indicating that there is no barrier between the haemolymph and the neuromuscular junctions (Irving et al., 1976).

Although the effect of ingested GABA on larval behaviour has not been examined, GABA injected directly into the haemolymph of OBLR larvae was shown to cause a decrease in the locomotory activity of the insect (R. Wong, personal communication). Lepidopteran saline, glutamate or GABA were injected hypodermically into fifth instar OBLR larvae between the seventh and eighth ventral segments. The final concentrations of glutamate and GABA in the haemolymph were 9.7 and 12.7 mM, respectively. Injections of saline or glutamate had no visible effect on the larvae. However, injections of GABA caused visible paralysis in the prolegs of the insect. Locomotory activity was quantified as the time required for the injected larvae to escape an arena after being placed in the center and allowed to ambulate freely. Larvae injected with saline or glutamate showed no significant difference in the amount of time required to escape the arena. However, insects injected with GABA showed a significant 2.3-fold increase in escape time indicating a decrease in locomotory activity. It is likely that the effect of injected GABA on the locomotory activity of insect larvae is a result of its inhibitory action in the insect nervous system.

Another consideration with respect to elevated GABA levels functioning as a plant resistance mechanism is the catabolic production cost. Many secondary plant substances which are thought to play roles in plant resistance have very high production costs (Schoonhoven, 1998). For example, terpenoids and alkaloids have production costs of 3.18 and 3.24, respectively, expressed as grams of glucose per gram of compound. Amino acids, however, have a relatively low production cost of
2.09. The total cost of chemical resistance depends on several factors including production cost, quantity produced, turnover rate and cost of transport and storage. A reduction in any of these costs will be beneficial to a plant as it has been demonstrated that increased amounts of secondary resistance compounds in plant tissues are correlated with a reduction in the plant biomass (Han and Lincoln, 1994). Therefore, the relatively low production cost of the amino acid GABA, and its utilization in the GABA shunt would favour its potential role as a plant resistance compound.

10.3.4 The Role of GABA in Host Plant Selection

Evidence has been provided which suggests that increased levels of GABA in leaf tissue may function as both a phagostimulant (Tables 2-3) and a feeding deterrent (Table 1). While the role of GABA as a phagostimulant implies a gustatory action, the role of feeding deterrent may be due to either gustatory or toxic neurophysiological action. Whether the feeding deterrency action is gustatory or neurophysiological there are potential explanations indicating why GABA may play an opposite role in different cases.

Considering the cases in which elevated GABA levels may promote or deter feeding through a gustatory action, GABA can be considered a semiochemical. It is known that the same semiochemical may play different roles under different chemical or environmental conditions. For example, the sesquiterpene hydrocarbon (E)-β-farnesene is released by aphids in response to predator attack or by plants in conjunction with the plant volatile (−)-β-caryophyllene. It can be detected by olfactory cells on aphid antennae. Detection of (E)-β-farnesene alone by an aphid causes rapid dispersal from the site of potential danger. However, detection of (E)-β-farnesene in conjunction
with (-)-β-caryophyllene does not elicit a response in aphids (Chamberlain et al., 2000). Thus plant derived semiochemicals may elicit different responses in insects depending on the nature of the chemical environment in which they occur. In the present study elevated GABA levels acted as a phagostimulant (Tables 2,3) in transgenic CaMVGADΔC tobacco plants exhibiting constitutively high GABA levels (Figs. 10,11) and as a feeding deterrent (Table 1) in transgenic CaMVGAD tobacco plants displaying elevated stress-induced accumulation of GABA (Figs. 8,9). The only effect of the CaMVGAD transgene appears to be an elevated stress-induced accumulation of GABA. In contrast it is clear that the CaMVGADΔC transgene has a pleiotropic effect on the physiology and morphology of the plant (Figs. 1, 2, 3, 4). Therefore, it is possible that differing chemical composition in the CaMVGAD and CaMVGADΔC strains causes GABA to act as a phagostimulant in one strain and as a feeding deterrent in the other. This could be due to differences in GABA levels and accumulation in the two strains. The constitutively high resting level of GABA present in CaMVGADΔC leaf tissue may be sufficient to cause phagostimulation during test-biting of leaf tissue while the relatively low resting level in CaMVGAD leaf tissue may not. Additionally, feeding deterrence in the CaMVGAD strain could be due to a combined effect of GABA and some other plant chemical whose occurrence may differ between the transgenic strains.

Considering the cases in which elevated GABA levels may or may not deter feeding through a toxic neurophysiological action, the differences in GABA levels and accumulation between the CaMVGAD and CaMVGADΔC tobacco strains may again play a factor. In order for ingested GABA to function as a neurophysiological toxin it must be absorbed from the gut into the haemolymph. Ingested CaMVGAD tissue may contain a relatively low resting level of GABA at the time of
consumption with the possibility that stress-induced GABA synthesis will occur in the gut. GABA synthesis would likely be due to Ca\(^{2+}\)/calmodulin-activation of GAD rather than pH-activation since the Lepidopteran midgut is strongly alkaline (pH ≈ 10) (Harvey and Nedergaard, 1964). GABA produced in the gut could then be absorbed into the haemolymph where it could act on the insect nervous system and result in reduced feeding by the insect. On the contrary, ingested CaMVGAD\(\Delta\)C tissue contains a constitutively high resting level of GABA at the time of consumption. It is possible that this high level of ingested GABA is detoxified in the gut before absorption into the haemolymph occurs. Thus differences in resting- and induced-levels of GABA between the CaMVGAD and CaMVGAD\(\Delta\)C tobacco strains may affect rates of GABA metabolism, retention, absorbance or excretion in the gut. This may account for the different results observed with the two strains.
10.4 Conclusions

The present study investigated the hypothesis that increased GABA levels in leaf tissue in response to insect activity may function as a plant resistance mechanism against phytophagous insect activity. Two testable predictions followed from this hypothesis:

1. Insect activity causes increased GABA levels in leaf tissue; and
2. Increased GABA levels in leaf tissue deter insects from feeding.

The first criterion was tested by subjecting leaf tissue to insect larval ambulatory activity and determining the subsequent GABA content of the leaf tissue. The criterion was supported by the finding that TBW larva ambulatory activity caused significant increases in the GABA content of tobacco leaf tissue (Fig. 12), and that OBLR larva ambulatory and leaf-rolling activity caused significant increases in the GABA content of soybean leaflets (Fig. 13).

The second criterion was tested by determining the phytophagous preference of insect larvae in two choice feeding preference assays using wt tobacco or transgenic tobacco with altered GABA metabolism. The criterion was not supported because mixed results were obtained. It was found that transgenic CaMVGAD tobacco leaves exhibiting an increased capacity for stress-induced GABA synthesis deterred TBW larvae from feeding. However, transgenic CaMVGADAC tobacco leaves exhibiting a higher constitutive level of GABA promoted feeding by TBW larvae.

Because of the mixed results obtained with respect to the second criterion, the hypothesis that increased GABA levels in leaf tissue in response to insect activity may function as a plant resistance mechanism against phytophagous insect activity is neither rejected nor accepted. Further work is required to understand the apparently contradictory results.
10.5 Further Experimentation

Further experimentation should be aimed at elucidating the factors which determine whether GABA functions as a phagostimulant or as a feeding deterrent. Analyzing the leaf cuticular layer to determine the GABA content on the surface of the leaf would be useful since palpation of the leaf surface is the first event in host-plant selection during which the insect receives chemical information directly from the plant. Two-choice preference assays between Samsun wt tobacco sprayed with either distilled water or GABA solution could be employed in order to determine whether GABA on the leaf surface affects host-plant selection. Analyzing the insect larval midgut contents, haemolymph and frass to determine their respective GABA contents could elucidate whether ingested GABA enters the midgut, and whether it is absorbed into the haemolymph or is excreted.
11. References


Mills WR, Joy KW. (1980) A rapid method for isolation of purified, physiologically active
chloroplasts, used to study the intracellular distribution of amino acids in pea leaves. *Planta* **148**:75-83.


**Shelp BJ, Walton CS, Snedden WA, Tuin LG, Oresnik IJ, Layzell DB.** (1995) GABA shunt in
REFERENCES

developing soybean seeds is associated with hypoxia. Plant Physiol. 94:219-228.


