Insect Footsteps on Leaves Stimulate the Accumulation of 4-Aminobutyrate and Can Be Visualized through Increased Chlorophyll Fluorescence and Superoxide Production[1]

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A substantial literature has demonstrated that within 2 to 3 h of insect herbivory or mechanical damage, plants synthesize wound-induced proteinase inhibitors that inhibit digestion (Bergey et al., 1996; Ryan, 2000). In contrast, we demonstrate here that the simple non-wounding crawling of insect larvae stimulates the synthesis of 4-aminobutyrate (GABA) within minutes, and that imprints of insect footsteps can be visualized within seconds through increases in chlorophyll fluorescence or superoxide production. We propose that the synthesis of GABA and superoxide represent rapidly deployed local resistance mechanisms that become operational before the local and systemic synthesis of proteinase inhibitors.

GABA is produced through an alpha-decarboxylation of L-Glu catalyzed by the cytosolic enzyme L-Glu decarboxylase (GAD; EC 4.1.1.15; Bown and Shelp, 1997). Plant GAD appears unique in possessing an autoinhibitory domain that can bind a Ca^{2+}/calmodulin complex (Snedden et al., 1996). When the resting level of cytosolic Ca^{2+} is raised, Ca^{2+}-/calmodulin-binding releases GAD inhibition and GABA is synthesized (Bown and Shelp, 1997). Plant cells exhibit increases in Ca^{2+} (Knight et al., 1991; Haley et al., 1995), GABA (Wallace et al., 1984; Ramputh and Bown, 1996), and superoxide concentrations (Harding et al., 1997) in response to non-wounding mechanical stimulation. Accumulation of GABA (Wallace et al., 1984; Ramputh and Bown, 1996) and superoxide (Felton et al., 1994; Bi and Felton, 1995; Ryan, 2000; de Bruxelles and Roberts, 2001) may function in plant resistance against herbivory. In animals, GABA is an inhibitory neurotransmitter (Sattelle, 1990; Casida, 1993). We investigated GABA accumulation, superoxide production, and changes in chlorophyll fluorescence in response to non-wounding mechanical stimulation of crawling insect larvae.

If GABA accumulation functions in induced resistance against insect herbivory, insect activity in leaf tissue should increase GABA concentrations. Five- and 10-min crawling of tobacco budworm (TBW; *Heliothis virescens* Fabricus) larvae on the upper surface of tobacco (*Nicotiana tabacum* cv Samsun NN) leaves raised GABA concentrations 4- and 5-fold, respectively (Table I). Similarly, 10-min crawling of oblique-banded leaf roller (OBLR; *Choristoneura rosaceana* Harris) larvae on soybean (*Glycine max* L. Merr. cv Harovinton) trifoliate leaflets resulted in a 10-fold increase in GABA concentrations over resting levels. OBLR larvae roll soybean leaflets into a protective tube using silk threads. Ten minutes of crawling followed by 10 min of leaf rolling resulted in an 18-fold GABA accumulation. OBLR larvae weighing 50 to 350 mg were employed, and significant GABA accumulation was observed when the weight was 140 mg or more (Table I). TBW larval crawling was restricted to one side of the midvein of a soybean leaflet for 10 min. The two halves of the leaflet were then separated for GABA analysis. A 15-fold GABA accumulation was observed in both halves of the leaflet. In contrast, the other two leaflets of the trifoliate did not accumulate GABA (Table I). Similar GABA accumulations were measured when larvae crawled under ambient laboratory light or in the dark.

GABA accumulation in response to crawling indicates a signaling process initiated by larval footsteps. Visualization of footsteps was sought by assessing chlorophyll fluorescence from plant-attached leaves in response to TBW activity. Increased chlorophyll fluorescence is indicative of reduced photosynthetic rates and plant cell stress (Lichtenthaler and Miehé, 1997; Chaerle and Van Der Straeten, 2000). In both tobacco and soybean, larval footsteps resulted in increased fluorescence that was detected within 20 s (Fig. 1, c and e). Typically, two parallel tracks resulting from a series of paired footsteps were visualized as corresponding areas of increased fluorescence (Fig. 1, c and e). Crawling resulted in four to 10 visualized footsteps per 10 s. Fluorescence was observed in soybean when TBW larvae weighing 40 mg or more were employed. In tobacco, the corresponding weight for the response was 140 mg. Chlorophyll

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Table I. *Insect crawling and leaf GABA concentrations*

Means ± SE are indicated. Within each of the experimental systems, means not sharing the same letter are significantly different. TBW larvae weighed 140 to 150 mg. OBLR larvae weighed 150 to 160 mg. TBW larvae weighed 140 to 150 mg.

Treatment	GABA	*n*	Significance
	nmol g^{-1} fresh wt		
Tobacco leaves and TBW larvae			
No crawling	12 ± 5	10	a
2-min Crawling	42 ± 11	10	a,b
5-min Crawling	56 ± 23	10	b
10-min Crawling	67 ± 20	10	b
Soybean leaflets and OBLR larvae			
No crawling	60 ± 18	17	a
10-min Crawling	669 ± 75	17	b
20-min Crawling	884 ± 89	6	b,c
10-min Crawling + 10-min leaf rolling	1,123 ± 123	11	c
Soybean leaflets and TBW larvae			
Control half leaflets, no crawling	50 ± 10	4	a
Half leaflet, 10-min crawling	810 ± 412	5	b
Corresponding half leaflet, no crawling	858 ± 532	5	b

fluorescence in response to footsteps was transient. In both tobacco and soybean, fluorescence intensity peaked 20 to 60 s after footsteps. In soybean, fluorescence declined to initial values within 5 and 30 min for larvae weighing 140 to 150 and 300 to 310 mg, respectively. Crawling did not result in footprints visible under white light. Insect herbivory also resulted in chlorophyll fluorescence at the edges of wounded tissue (Fig. 1, k and l). This response was observed within 20 s of herbivory and a halo of fluorescence appeared around the head of the chewing insect (Fig. 1k). Fluorescence in response to wounding remained after fluorescence in response to footsteps returned to control levels (Fig. 1l).

Accumulation of superoxide and reactive oxygen species has been implicated in plant resistance against herbivory (Felton et al., 1994; Bi and Felton, 1995; Ryan, 2000; de Bruxelles and Roberts, 2001). To detect superoxide production in response to footsteps, leaves were excised immediately after crawling, and vacuum infiltrated with a solution containing nitroblue tetrazolium (NBT). Superoxide reduces soluble NBT to a purple precipitate that results in tissue staining. Staining in response to footsteps was observed in both tobacco and soybean with larvae weighing 50 mg or more. Larval footsteps resulted in corresponding areas of tissue staining and increased chlorophyll fluorescence (Fig. 1, e–h). To determine the timing of superoxide production, tobacco and soybean leaves were excised and infiltrated with NBT before crawling. Staining in response to footsteps was detected within 10 s. Fluorescence data could not be obtained from infiltrated tissue due to high levels of chlorophyll fluorescence (possibly due to lower diffusion of CO_2 in liquid, and consequent inhibition of photosynthesis). Thus, the temporal sequence of changes in fluorescence and tissue staining could not be determined. To determine the duration of the response, leaf excision and NBT infiltration were delayed for set intervals after crawling. Staining could be detected in tobacco leaf tissue for up to 72 h and in soybean leaf tissue for up to 20 h. Increased fluorescence and tissue staining were also detected after crawling had occurred in the dark, and when upside-down TBW larvae crawled on the lower leaf surface. Simultaneous infiltration with NBT and superoxide dismutase, which converts superoxide to hydrogen peroxide, eliminated tissue staining in both tobacco and soybean. In contrast, catalase, which converts hydrogen peroxide to water and oxygen, did not eliminate staining. These data demonstrate that tissue staining results from superoxide production. Corresponding areas of increased chlorophyll fluorescence and superoxide production were also observed at the edges of wounded tissue after herbivory (Fig. 1, l and m). The same responses were observed after suction was applied with a micropipette tip with an external diameter of 0.5 mm (Fig. 1, i and j). These observations, plus data indicating a minimal weight for footstep visualization, demonstrate that the responses are initiated by mechanical stimulation, not insect-derived signal molecules.

Leaves are subject to contact with raindrops, wind, and wind-borne inanimate objects. However, when soybean or tobacco plants were placed in the wind or rain, increases in chlorophyll fluorescence or superoxide production were not observed. Thus, the responses to larval crawling can be attributed to the mechanism of crawling (Chapman, 1969). TBW larvae are typical in possessing eight pairs of legs. These are cylindrical outgrowths of the soft body wall, and have a lumen that is continuous with the body fluid. Crawling results from muscles acting on the incompressible body fluid to extend or contract body shape, in combination with feet that grip the substratum. Feet have a circular apical area known as the planta, which can function as a sucker when its center is drawn up by a retractor muscle to create a vacuum. Thus, an insect planta will exert negative central and positive peripheral pressures on the leaf surface when it anchors the larva. These pressures will result in tensions in the underlying tissue that, dependent on insect activity, will be maintained for varying periods of time. Visualized footsteps had a diameter of approximately 0.5 mm (Fig. 1, g and h), closely corresponding to the diameter of the larval planta. In contrast, wind and rain will not create suction, and contact time with inanimate objects will be brief. Larval footsteps and suction applied with a micropipette resulted in similar patterns, with fluorescence (Fig. 1i) and staining (Fig. 1j) more apparent at the periphery. Mechanical wounding of leaf tissue has been shown to inhibit photosynthesis (Herde et

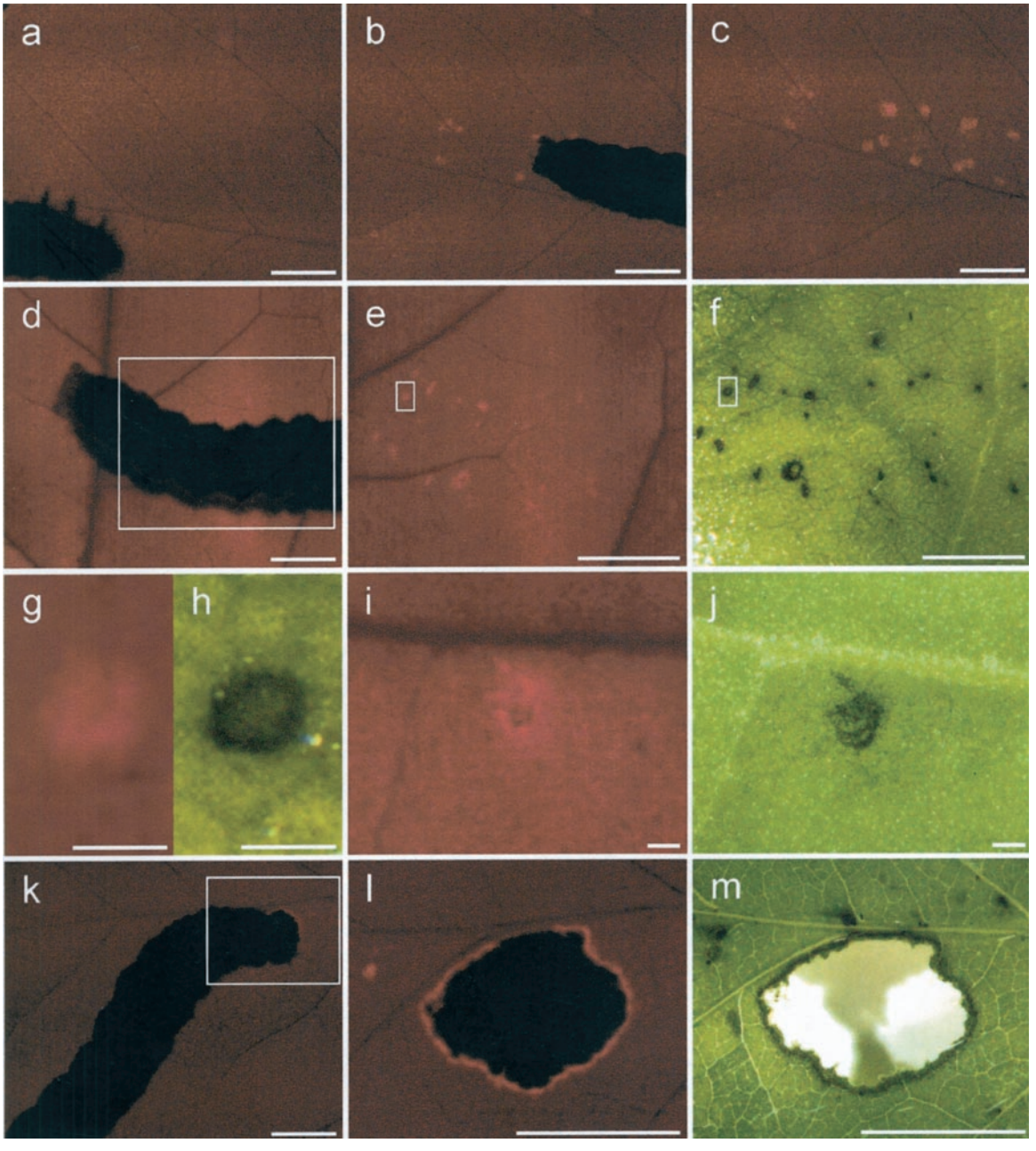

Figure 1. Chlorophyll fluorescence and superoxide detection in response to herbivory or crawling. a through c, TBW larva traversing a tobacco leaf from left to right at 0, 110, and 120 s, respectively, and two parallel tracks of resulting fluorescent footsteps (b and c). d through f, Result of TBW larva moving left to right across a tobacco leaf (d), and enlarged images of fluorescent footsteps 50 s later (e) and of corresponding areas of superoxide production (f). g and h, Enlarged images of a footstep from e and f. i and j, Corresponding fluorescence (i) or superoxide production (j) after suction applied with a micropipette tip. k through m, TBW larva feeding on a soybean leaflet (k), and enlarged images indicating fluorescence around the perimeter of the lesion 5 min later (l) and corresponding superoxide production (m). (Reflected green light is observed within the lesion.) Scale bars indicate 5 mm, except in g through j, where they indicate 0.5 mm. Boxes indicate areas of enlargement.

al., 1999). However, to our knowledge, increased chlorophyll fluorescence or superoxide production in response to insect footsteps has not been documented. Plant cell responses to mechanical stimuli have been attributed to increases in cytosolic Ca^{2+} (Knight et al., 1991; Haley et al., 1995) mediated by plasma membrane-located stretch-activated Ca^{2+} channels (Zimmermann et al., 1997). Similarly, increased Ca^{2+} levels may mediate responses to insect footsteps.

Growth, development, and survival of OBLR larvae are reduced by artificial diets containing elevated

GABA levels (Ramputh and Bown, 1996). Many insecticides are agonists or antagonists of the GABA-gated Cl^- channel, and disrupt neuromuscular activity when absorbed through the cuticle (Sattelle, 1990; Casida, 1993). Insect larval neuromuscular junctions are not protected by a layer of glial cells, and injection of neurotransmitters into hemolymph causes reversible paralysis (Irving et al., 1976, 1979). Leaf GABA levels rise rapidly in response to mechanical stimulation (Wallace et al., 1984; Ramputh and Bown, 1996), damage (Wallace et al., 1984; Ramputh and Bown, 1996), or insect crawling (Table I). Thus, disruption of neuromuscular activity may result from GABA accumulation and ingestion during herbivory. GABA accumulation in response to herbivory could not be determined because sampling of the wounded tissue, in itself, will cause rapid GABA accumulation. Accumulation in response to crawling is consistent with the hypothesis that GABA functions in resistance against insect herbivory.

In tomato, identical responses to insect herbivory and experimentally applied leaf wounding have been documented over the past 30 years (Green and Ryan, 1972; Bergey et al., 1996; Ryan, 2000). An initial response to either is the production of systemin, an 18-amino acid signal polypeptide, through proteolytic cleavage of prosystemin in the affected tissue. Systemin stimulates the localized and systemic production of a variety of defense proteins, including proteinase inhibitors, which block digestion and deter insect feeding. Proteinase inhibitors have been detected 2 h or more after herbivory or wounding. However, GABA accumulation within 5 min (Table I) and increased chlorophyll fluorescence and superoxide production within 20 s (Fig. 1, c, e, f, l, and m) demonstrate much faster responses to both non-wounding insect crawling and feeding. Increased chlorophyll fluorescence is associated with an inhibition of photosynthesis and occurs in response to various stresses (Lichtenthaler and Miehé, 1997; Chaerle and Van Der Straeten, 2000). Oxidative enzymes and reactive oxygen species accumulate in response to wounding (Orozco-Cardenas and Ryan, 1999; de Bruxelles and Roberts, 2001) and herbivory (Felton et al., 1994; Bi and Felton, 1995). In addition, when corn earworm (*Helicoverpa zea*) larvae consumed leaf tissue from soybean plants previously damaged by herbivory, decreased growth rates and oxidative damage to the midgut of the larvae were observed (Felton et al., 1994; Bi and Felton, 1995). As a consequence, reactive oxygen species appear to have a direct role in resistance against herbivory.

Thus, the prevailing models of induced plant resistance to insect herbivory (Bergey et al., 1996; Ryan, 2000; de Bruxelles and Roberts, 2001) require modification to include responses, which are: (a) too rapid to be dependent on gene expression, and (b) initiated by non-wounding insect crawling. Accumulation of GABA and superoxide may represent rapidly deployed, local resistance mechanisms that become operational before the synthesis of proteinase inhibitors. Whether superoxide production in response to crawling can trigger a systemic response remains to be determined.

OBLR and TBW egg masses were hatched and individual larvae placed in containers with an omnivorous leaf roller diet (Bio-Serv Inc., Frenchtown, NJ) or an artificial TBW diet (Southland Products Inc., Lake Village, AR), respectively. They were grown at 22°C under a 16-h-light/8-h-dark cycle. Soybean and tobacco seeds were germinated and grown in a greenhouse at 25°C to 30°C under natural light (maximum 500–600 μmol s^{-1} m^{-2}) from March to November. They were watered every other day, and tobacco plants fertilized weekly with Peters Professional 21-7-7 (N-P-K) Acid Fertilizer (Scotts-Sierra Horticultural Products Company, Marysville, OH). Tobacco plants were employed at the seven-leaf stage, and leaf number 5 was used for experimental manipulations. The first trifoliate of soybean plants was utilized as the second trifoliate was emerging. To avoid GABA accumulation in response to mechanical manipulation, plant-attached leaves, leaflets, or half leaflets were excised directly into liquid nitrogen.

GABA was extracted and determined spectrophotometrically using a coupled enzyme assay (Zhang and Bown, 1997).

For fluorescence measurements, plant-attached leaves were placed horizontally on a black surface and illuminated using an EXR 300W photo lamp (Wiko Ltd., Orland Park, IL). Light was passed through a blue filter (425–525 nm), and leaves were exposed to a light intensity of 140 μmol s^{-1} m^{-2}. Red chlorophyll fluorescence was passed through a red filter (700 nm and above) and detected by a COHU 4915–2001/0000 High Performance CCD camera (Imaging Research, Inc., St. Catharines, ON). Before larval crawling, herbivory, or other treatments, leaves were illuminated for 7 min to reach steady-state fluorescence. Images were recorded at 10-s intervals and relative fluorescence from individual footsteps was analyzed as a function of time using Analytical Imaging Station software (Imaging Research, Inc.).

Superoxide was detected using an adaptation of a published method (Jabs et al., 1996). After larval crawling, herbivory, or other treatments, leaves were excised and vacuum infiltrated for 20 min with 0.1% (w/v) NBT, 10 mM NaN_3, and 10 mM KH_2PO_4 buffer (pH 7.8), and incubated for a further 20 min. NBT is reduced to a dark purple formazan precipitate in the presence of superoxide. When used, superoxide dismutase (azide insensitive; 218 units mL^{-1}) or catalase (1360 units mL^{-1}; Sigma-Aldrich Canada Ltd., Oakville, ON) were added directly to the reaction mixture before infiltration. Infiltrated leaves were placed on a horizontal white surface and viewed using a Zeiss Stemi SV 11 dissecting microscope (Carl Zeiss Vision GmbH, Munich-Hallbergmoos, Germa-

ny). Digital images were captured using a Zeiss SoundVision SV Micro digital camera and recorded using Zeiss AxioVision software.

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