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Short communication

Footsteps from insect larvae damage leaf surfaces and initiate rapid responses

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Abstract

Plant resistance to insect herbivory involves gene expression in response to wounding and the detection of insect elicitors in oral secretions (Kessler and Baldwin, 2002, Ann. Rev. Plant Biol. 53: 299–328). However, crawling insect larvae stimulate the synthesis of 4-aminobutyrate within minutes and imprints of larval footsteps can be visualized within seconds through superoxide production or transient increases in chlorophyll fluorescence (Bown et al., 2002, Plant Physiol. 129: 1430–1434). Here cryo-scanning electron microscopy was used to demonstrate that larval feet, which are equipped with a perimeter row of hook-like crochets, damage leaf tissue and result in larval footprints. Staining for cell death shows that areas of wounding correspond to footsteps detected through increased chlorophyll fluorescence. Superoxide production in response to footsteps was inhibited by diphenyleneiodonium, an inhibitor of the plasma membrane NADPH oxidase enzyme. Inhibition of superoxide production, however, did not eliminate the detection of cell death. The results demonstrate that larval footsteps damage leaf tissue, and initiate rapid local responses which are not dependent on herbivory or oral secretions. It is proposed that superoxide production at the wound site prevents opportunistic pathogen infection.

Abbreviations: cryo-SEM – cryo-scanning electron microscopy; DPI – diphenyleneiodonium; GABA – 4-aminobutyrate; NBT – nitroblue tetrazolium.

Plants respond to herbivore attack with a variety of induced responses which may be specific to elicitors in insect oral secretions or a more general wound response. Defence-related genes are activated through a signalling cascade and hours may elapse before defence proteins are synthesized (Ryan, 2000; Kessler and Baldwin, 2002). The wound response involves the production of reactive oxygen species, including superoxide and its product hydrogen peroxide (Kessler and Baldwin, 2002). Superoxide is produced by a plasma membrane located NADPH oxidase (Desikan et al., 1996; Keller et al., 1998), which can be inhibited by diphenyleneiodonium (DPI) (Jabs et al., 1996). Hydrogen peroxide is produced both locally and systemically in the plant within 1 h of wounding (Orozco-Cardenas and Ryan, 1999). The wound response also involves the proteolytic cleavage of prosystemin into systemin (Ryan, 2000; Kessler and Baldwin, 2002). Systemin binds to a plasma-membrane located receptor (Ryan, 2000), initiating a signalling cascade which leads to the release of linoleic acid from the plasma membrane (Conconi et al., 1996). Linoleic acid is converted into jasmonic acid which accumulates locally in the plant within 2 h of wounding (Creelman et al., 1992). This signalling cascade leads to the activation of wound-induced defence genes within hours (Ryan, 2000). For example, 6 h after wounding tomato leaves show increased local expression of a calcium-dependent protein kinase (Chico et al., 2002).

In contrast, recent experiments investigated the responses of soybean (*Glycine max*) and tobacco (*Nicotiana tabacum*) leaves to crawling tobacco

budworm (TBW) larvae (*Heliothis virescens*). Crawling in the absence of herbivory, resulted in footsteps visualized in seconds through increases in chlorophyll fluorescence or superoxide production. 4-aminobutyrate (GABA) accumulation was detected within minutes (Bown et al., 2002). GABA is a four-carbon, non-protein amino acid which in invertebrates is an inhibitory neuromuscular transmitter (Sattelle, 1990; Casida, 1993). In plants, GABA accumulates in response to many stresses including mechanical stimulation and mechanical damage (Wallace et al., 1984; Ramputh and Bown, 1996).

The present paper investigates the mechanisms underlying the rapid responses observed following larval crawling on soybean and tobacco. It was originally suggested that responses to crawling resulted from non-damaging suction pads on larval feet (Bown et al., 2002). The present paper, however, demonstrates that superoxide production is initiated after larval footsteps damage leaf tissue.

Tobacco budworm (*H. virescens*) egg masses were obtained and reared (Bown et al., 2002). Tobacco (*N. tabacum* cv Samsun NN) and soybean (*Glycine max* cv. Harovinton) seeds were planted and grown (Bown et al., 2002). Soybean plants were grown to the first trifoliate stage and a fully expanded first trifoliate was used for experimentation. Tobacco plants were grown to the 7-leaf stage and leaves number 4 and 5 were used for experimental purposes. Plants were transported into the laboratory at least 1 h before use and placed under a 105–150 $\mu mol\, s^{-1}\, m^{-2}$ intensity SunMaster 400 W halogen lamp (PGL Group/Venture, USA). When leaves were examined for responses to nitroblue tetrazolium (NBT) or Evan's blue stains at timed intervals after crawling, plants were maintained in the laboratory prior to leaf excision and staining.

Cryo-scanning electron microscopy (cryo-SEM) was conducted at Wageningen University (the Netherlands) using a field-emission SEM (Jeol 6300F), equipped with an Oxford CT 1500 HF cryo system. Leaf and larva samples were mounted on a sample holder with conductive carbon cement and rapidly frozen in liquid nitrogen. The surfaces were freeze-etched for 15 min at $-89\,^{\circ}C$ and 10^{-4} Pa and sputter-coated with platinum. The samples were analyzed with an accelerating voltage of 2.5 kV and photographed using a digital imaging system.

Lepidopteran larvae are known to have prolegs equipped with outwardly curved hooks known as crochets (Chapman, 1969). Cryo-SEM images demonstrate that the cabbage moth (*Mamestra brassicae*) larval proleg is typical, having a flat, circular planta, equipped with a peripheral row of crochets (Figure 1a). Cryo-SEM was used to examine leaf surfaces after larval crawling. Resulting damage to the leaf surface was approximate in size to the planta and associated crochets (Figure 1a and b). Damage includes the peeling back of the cuticle and the rupturing of cells immediately in contact with the planta (Figure 1b). Surrounding the damaged cells are additional cells that have lost turgor (Figure 1b). In contrast to the circular area of damage caused by the prolegs (Figure 1b), the thoracic legs (Figure 1c) of the larva have a terminal claw and cause a thin slit in the leaf (Figure 1d). Larvae mechanically restrained so that only the thoracic legs contacted the tobacco leaf caused slit damage visualized with staining for cell death and superoxide production (data not shown). Thus both the thoracic legs and the prolegs can cause leaf damage.

Histological staining with Evan's blue was used to detect cell death in tobacco and soybean in response to larval crawling. Evan's blue staining for cell death was performed without vacuum infiltration using excised leaves. Evan's blue dye is unable to cross intact membranes, thus live cells exclude the dye, while dead cells stain blue (Gaff and Okong'O-Ogola, 1971). To determine the time limit of detection for Evan's blue staining, leaves were incubated for 40 min in a 0.05% w/v solution of Evan's blue (Sigma-Aldrich Canada Ltd., Oakville, Ontario, Canada) at 0 h, 30 min, 1 h, 4 h, 1–5, 7–10, 15 d subsequent to crawling. For all trials other than the time course, leaves were incubated immediately after insect crawling. After rinsing for 1 min with water leaves were imaged using a Zeiss Stemi SV 11 dissecting microscope (Carl Zeiss Vision GmbH, München-Hallbergmoos, Germany). Digital images were captured using a Zeiss SoundVision SV Micro digital camera and recorded using Zeiss Axiovision software.

Fluorescent imaging of plant-attached leaves in response to larval crawling was conducted as indicated previously (Bown et al., 2002). Within seconds of crawling (Figure 2a and b), footsteps were visualized as a transient increase in chlorophyll fluorescence (Figure 2c and d). Subsequent staining indicated that cell death occurs in areas corresponding to those exhibiting increased chlorophyll fluorescence (Figure 2e and f). Thus leaf damage in response to larval footsteps can be observed using both

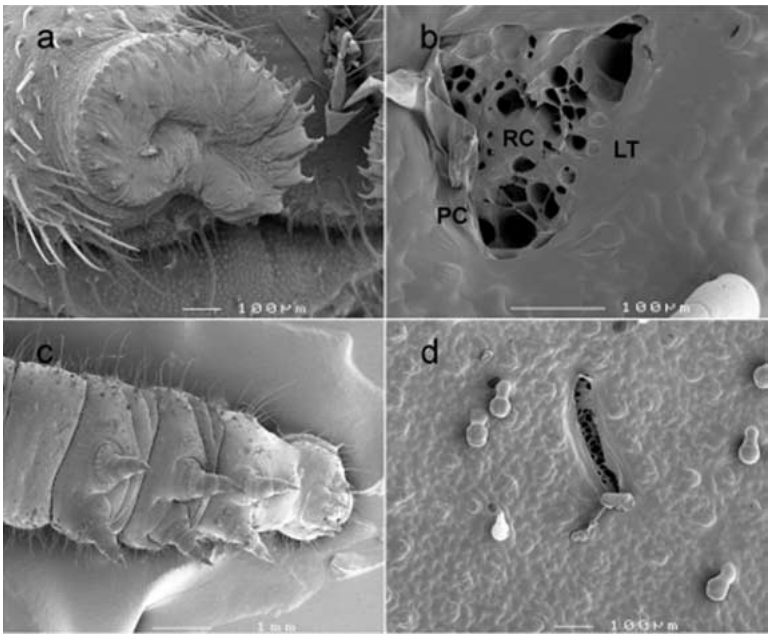

Figure 1. Cryo-SEM images of larval feet and associated damage to leaf tissue. Panels (a) and (b) show a cabbage moth larva proleg, and damage to the leaf surface following crawling. LT indicates cells which have lost turgor, RC indicates ruptured cells and PC indicates the peeling back of the cuticle. Panels (c) and (d) show the thoracic legs of the larva and the corresponding damage to the leaf resulting from crawling.

cryo-SEM (Figure 1) and histological staining techniques (Figure 2).

After crawling, superoxide production in excised leaves was monitored with NBT and recorded (Bown et al., 2002), except that leaves were incubated in NBT without vacuum infiltration. DPI chloride (Sigma-Aldrich Canada Ltd., Oakville, Ontario, Canada) was used to inhibit superoxide production. Immediately following chlorophyll fluorescence imaging in response to larval crawling, leaves were excised into 50 μM DPI in 1% DMSO. They were vacuum infiltrated for 40 min and incubated for a further 20 min. Although, vacuum infiltration of NBT was not required for superoxide staining, infiltration with DPI was required to block NBT staining. This suggests that superoxide was more diffuse and accessible to NBT than the membrane-bound NADPH oxidase to DPI. Leaves were then immediately stained for superoxide production.

Larval footsteps on soybean and tobacco stimulate superoxide production (Bown et al., 2002; Figure 3). The previous demonstration of superoxide production in response to footsteps involved vacuum infiltration of NBT. In contrast, the present study found that staining resulted when leaf tissue was incubated with NBT without vacuum infiltration. Corresponding patterns of NBT staining and fluorescent footsteps were seen after larval crawling (Figure 3). In both soybean and tobacco superoxide production in response to crawling was inhibited by subsequent incubation with DPI (Figure 3b). However, control leaves incubated in solvent minus DPI exhibited normal NBT staining (Figure 3c and d). Conversely, tobacco and soybean leaves still stained for cell death when crawling was followed by DPI treatment (Figure 3e and f).

After crawling, staining for cell death was detected within 30 min and was detectable for up to 10 d in tobacco and up to 5 d in soybean. Superoxide production was maintained for 1–3 d after crawling in both tobacco and soybean leaves. TBW larva weighing only 7 mg caused a transient increase in chlorophyll fluorescence in soybean, whereas in tobacco, chlorophyll fluorescence was observed with larvae weighing 60 mg or more. Larvae weighing 40 mg or more were necessary for superoxide production and cell death in soybean. Corresponding values for tobacco were 40 mg for cell death and 25 mg for superoxide production.

Cryo-SEM images demonstrate that the crochets and planta at the distal end of the larval proleg damage the

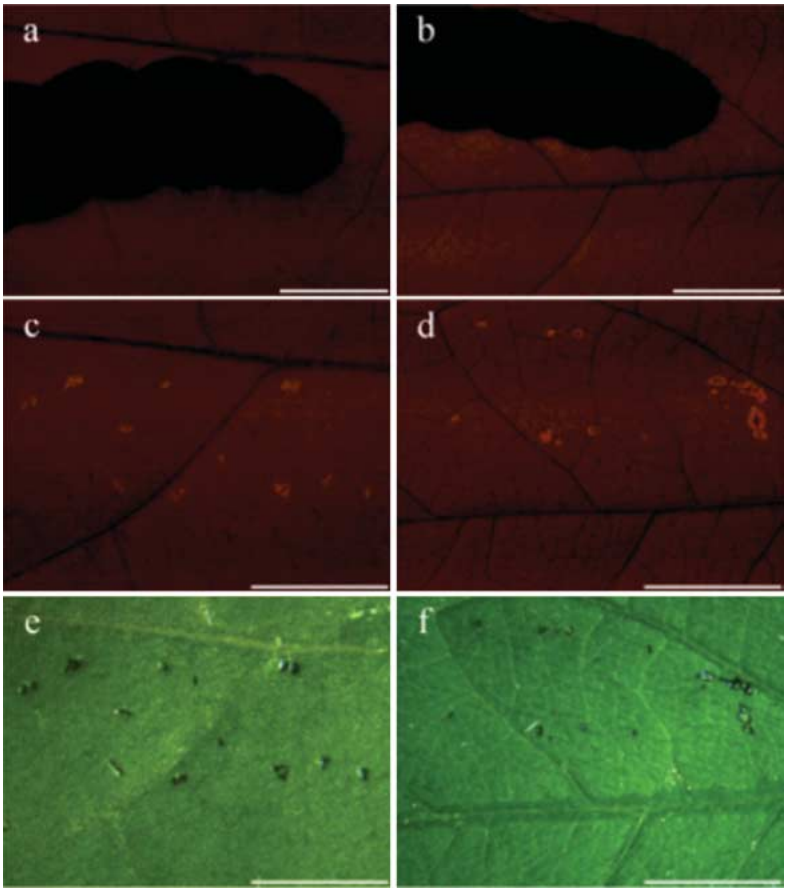

Figure 2. Larval footsteps result in increased chlorophyll fluorescence and corresponding cell death. Panels (a) and (b) show TBW larvae crawling from left to right across tobacco and soybean leaves respectively. Panels (c) and (d) show two tracks of fluorescent footsteps on tobacco (c) and soybean (d). Panels (e) and (f) show corresponding Evan's blue staining for cell death on tobacco (e) and soybean (f). Scale bars indicate 5 mm.

leaf surface (Figure 1). Larvae move through hydrostatic pressure exerted by longitudinal muscles on the haemolymph. Larval crawling is initiated by the posterior anal claspers moving forward and attaching to the leaf surface. Muscle contractions cause forward movements of each pair of prolegs. This continues until each pair of legs has obtained a fresh grip on the leaf surface. When a proleg comes in contact with a leaf, the crochets engage and the planta of the larva contacts the leaf surface. A retractor muscle in the proleg then contracts causing suction between the planta and the leaf, and disengagement of the crochets from the leaf tissue (Chapman, 1969). Different types of damage are caused by the thoracic legs and prolegs, respectively (Figure 1). As a larva crawls across the leaf, the prolegs release the suction and disengage the crochets, causing circular damage to the leaf surface (Figure 1b). This is contrary to the slit-like damage caused by the thoracic legs as the larva grips the leaf (Figure 1d). Damage to leaf surfaces was also demonstrated with visualization of footsteps using a stain for cell death (Figure 2).

Areas of superoxide production correspond to a transient increase in chlorophyll fluorescence (Bown et al., 2002; Figure 3). Previous reports used vacuum infiltration of NBT to stain for superoxide (Bown et al., 2002). Here, however, vacuum infiltration was not used. Thus the data demonstrate that the structural integrity of the leaf epidermal layer was broken and that superoxide produced at the wound site is exposed to externally applied NBT. When leaves were treated with DPI, NBT staining in response to superoxide was barely visible (Figure 3b). DPI is known to inhibit the plasma-membrane located NADPH oxidase (Desikan et al., 1996; Jabs et al., 1996; Keller et al., 1998).

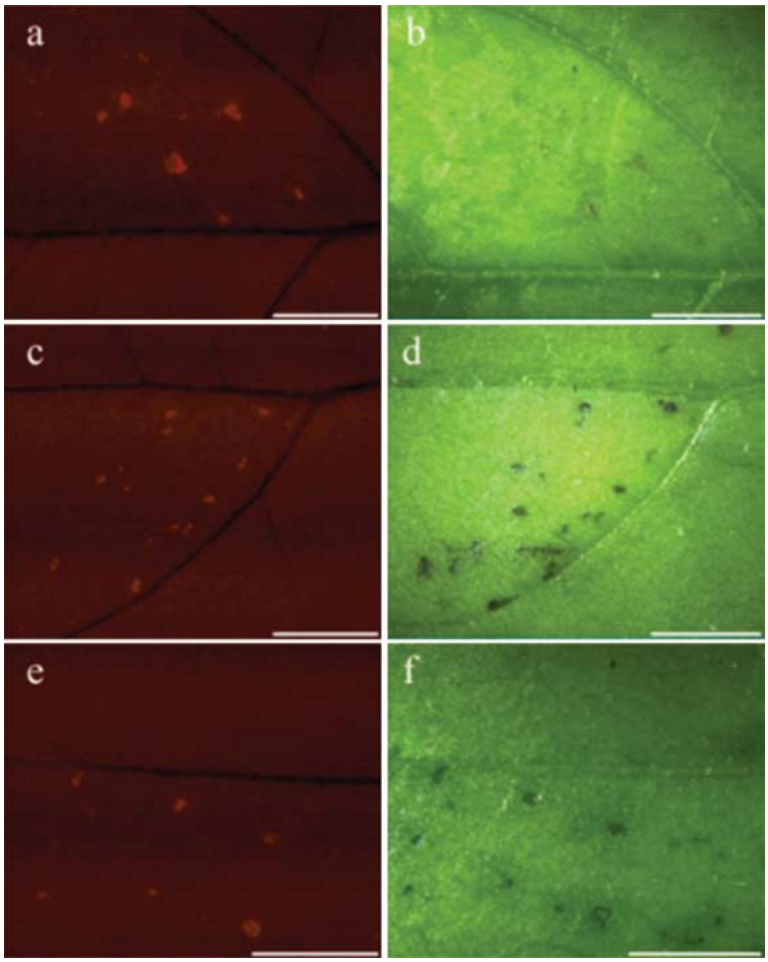

Figure 3. DPI inhibits superoxide production in response to larval footsteps but does not inhibit detection of cell death. Panel (a) shows fluorescent footsteps resulting from a TBW larva which crawled diagonally from bottom right to top left of a tobacco leaf. Panel (b) shows DPI inhibition of superoxide production on the same leaf, as indicated by the absence of NBT staining. Panel (c) shows fluorescent footsteps resulting from TBW crawling from bottom left to top right on a tobacco leaf. Panel (d) shows the same leaf and that infiltration with the solvent minus DPI does not inhibit superoxide production. Panel (e) shows fluorescent footsteps resulting from TBW larva crawling from left to right across a tobacco leaf. Panel (f) shows the same leaf and that Evan's blue staining for cell death is not inhibited when the leaf is treated with DPI. Scale bars indicate 5 mm.

This suggests that the source of superoxide production is the plasma-membrane located NADPH oxidase, which is activated in response to wounding (Orozco-Cardenas and Ryan, 1999). This conclusion is also supported by the elimination of NBT visualized footsteps when leaves were incubated with superoxide dismutase (Bown et al., 2002). However, Evan's blue staining for cell death was not eliminated when tissue was treated with DPI immediately after larval crawling (Figure 3f). Thus, some cell death occurs in response to larval crawling, not superoxide production. The minimum weight required for chlorophyll fluorescence, superoxide production and cell death indicates that the mechanism involves cell damage, not an insect-derived signal molecule.

The role of superoxide production in response to larval footstep damage may be three-fold. First, superoxide is converted into hydrogen peroxide via the enzyme superoxide dismutase (Hammond-Kosack and Jones, 1996). Hydrogen peroxide can diffuse across membranes and function as a signal molecule to initiate responses to wounding

(Orozco-Cardenas and Ryan, 1999). Second, ingestion of previously wounded tissue and associated reactive oxygen species results in oxidative damage to the midgut of insects, stunts growth and deters subsequent feeding (Felton et al., 1994; Bi and Felton, 1995; Bi et al., 1997). Third, whereas signal molecules are generally transient, superoxide production is maintained for 1–3 d following crawling. Wound-induced superoxide production is well documented (Kessler and Baldwin, 2002). Thus the present paper which demonstrates damage at the footstep site indicates that the long-term production of reactive oxygen species may function as an antiseptic mechanism to guard against opportunistic pathogen infection at the exposed wound site. Superoxide and associated hydrogen peroxide are produced in response to viral, bacterial and fungal pathogens, and are believed to deter infection (Wojtaszek, 1997; Lüthje et al., 2000).

Larvae crawling on leaves cause a rapid and large increase in GABA levels (Bown et al., 2002). Here we show that larval footsteps cause damage to the leaf surface (Figures 1 and 2). Rapid and large GABA increases in response to mechanical stimulation or mechanical damage to soybean leaves have been demonstrated previously (Wallace et al., 1984; Ramputh and Bown, 1996). However, accumulation in response to mechanical damage is much greater than that in response to mechanical stimulation. Thus the rapid and large increases in GABA seen in response to larval crawling (Bown et al., 2002) appear to result from damage to the leaf by larval footsteps. GABA, an inhibitory neurotransmitter may function against invertebrate pests when ingested. A recent paper has shown that overexpression of glutamate decarboxylase in transgenic tobacco plants confers resistance against the northern root-knot nematode (McLean et al., 2003). Thus, induced GABA accumulation may represent a common plant response to invertebrate attack and result in rapidly deployed local resistance (Bown et al., 2002) that becomes operational before the expression of wound-induced defence genes (Ryan, 2000).

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References

Bi JL and Felton GW (1995) Foliar oxidative stress and insect herbivory: Primary compounds, secondary metabolites, and reactive oxygen species as components of induced resistance. Journal of Chemical Ecology 21: 1511–1530.

Bi JL, Murphy JB and Felton GW (1997) Antinutritive and oxidative components as mechanisms of induced resistance in cotton to *Helicoverpa zea*. Journal of Chemical Ecology 23: 97–117.

Bown AW, Hall DE and MacGregor KB (2002) Insect footsteps on leaves stimulate the accumulation of 4-aminobutyrate and can be visualized through increased chlorophyll fluorescence and superoxide production. Plant Physiology 129: 1430–1434.

Casida JE (1993) Insecticide action at the GABA-gated chloride channel: Recognition, progress and prospects. Archives of Insect Biochemistry and Physiology 22: 13–23.

Chapman RF (1969) Locomotion. In: The Insects. American Elsevier Publishing Company, Inc., New York, pp. 154–157.

Chico JM, Raíces M, Téllez-Iñón MT and Ulloa RM (2002) A calcium-dependent protein kinase is systemically induced upon wounding in tomato plants. Plant Physiology 128: 256–270.

Conconi A, Miquel M, Browse JA and Ryan CA (1996) Intracellular levels of free linolenic and linoleic acids increase in tomato leaves in response to wounding. Plant Physiology 111: 797–803.

Creelman RA, Tieney ML and Mullet JE (1992) Jasmonic acid/methyl jasmonate accumulate in wounded soybean hypocotyls and modulate wound gene expression. Proceeding of the National Acadamy of Science USA 89: 4938–4941.

Desikan R, Hancock JT, Coffey MJ and Neill SJ (1996) Generation of active oxygen in elicited cells of *Arabidopsis thaliana* is mediated by a NADPH oxidase-like enzyme. FEBS Letters 382: 213–217.

Felton GW, Bi JL, Summers CB, Mueller AJ and Duffey SS (1994) Potential role of lipoxygenases in defense against insect herbivory. Journal of Chemical Ecology 20: 651–666.

Gaff DF and Okong'O-Ogola O (1971) The use of non-permeating pigments for testing the survival of cells. Journal of Experimental Botany 22: 756–758.

Hammond-Kosack KE and Jones JDG (1996) Resistance gene-dependent plant defense responses. Plant Cell 8: 1773–1791.

Jabs T, Dietrich RA and Dangl JL (1996) Initiation of runaway cell death in an *Arabidopsis* mutant by extracellular superoxide. Science 273: 1853–1856.

Keller T, Damude HG, Werner D, Doerner P, Dixon RA and Lamb C (1998) A plant homolog of the neutrophil NADPH oxidase $gp91^{phox}$ subunit gene encodes a plasma membrane protein with Ca^{2+} binding motifs. Plant Cell 10: 255–266.

Kessler A and Baldwin IT (2002) Plant responses to insect herbivory: The emerging molecular analysis. Annual Review of Plant Biology 53: 299–328.

Lüthje S, Böttger M and Döring O (2000) Are plants stacked neutrophiles? Comparison of pathogen-induced oxidative burst in plants and mammals. Progress in Botany 61: 187–222.

McLean MD, Yevtushenko DP, Deschene A, Cauwenberghe ORV, Makhmoudova A, Potter JW, Bown AW

and Shelp BJ (2003) Transgenic tobacco plants overexpressing glutamate decarboxylase are resistant to the root-knot nematode. Molecular Breeding 11: 277–285.

Orozco-Cardenas M and Ryan CA (1999) Hydrogen peroxide is generated systemically in plant leaves by wounding and systemin via the octadecanoid pathway. Proceedings of National Acadamy of Science USA 96: 6553–6557.

Ramputh AI and Bown AW (1996) Rapid γ-aminobutyric acid synthesis and the inhibition of the growth and development of oblique-banded leaf-roller larvae. Plant Physiology 111: 1349–1352.

Ryan CA (2000) The systemin signaling pathway: Differential activation of plant defensive genes. Biochimica and Biophysica Acta 1477: 112–121.

Sattelle DB (1990) GABA receptors of insects. Advances in Insect Physiology 22: 1–113.

Wallace W, Secor J and Schrader LE (1984) Rapid accumulation of γ-aminobutyric acid and alanine in soybean leaves in response to an abrupt transfer to lower temperature, darkness, or mechanical manipulation. Plant Physiology 75: 170–175.

Wojtaszek P (1997) Oxidative burst: An early plant response to pathogen infection. Biochemical Journal 322: 681–692.