

# Caterpillar salivary enzymes: "eliciting" a response

## Enzymes salivaires de chenille : la « stimulation » d'une réaction

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### Article abstract

Plants exhibit remarkable plasticity in their ability to differentiate between herbivorous insect species and subtly adjust their defense responses to target distinct pests. One key mechanism used by plants to recognize herbivorous caterpillars is elicitors present in their oral secretions; however, these elicitors not only cause the induction of plant defenses but recent evidence suggests that they may also suppress plant responses. The absence of "expected changes" in induced defense responses of insect-infested plants has been attributed to hydrogen peroxide produced by caterpillar salivary glucose oxidase (GOX). Activity of this enzyme is variable among caterpillar species; it was detected in two generalist caterpillars, the beet armyworm (*Spodoptera exigua*) and the bertha armyworm (*Mamestra configurata*), but not in other generalist or specialist caterpillar species tested. In the beet armyworm, GOX activity fluctuated over larval development with high activity associated with the salivary glands of fourth instars. Larval salivary GOX activity of the beet armyworm and the bertha armyworm was observed to be significantly higher in caterpillars reared on artificial diet as compared with those reared on *Medicago truncatula* plants. This implies that a factor in the diet is involved in the regulation of caterpillar salivary enzyme activity. Therefore, plant diet may be regulating caterpillar oral elicitors that are involved in the regulation of plant defense responses: our goal is to understand these two processes.

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Magali Merkx-Jacques and Jacqueline C. Bede<sup>1</sup>

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Plants exhibit remarkable plasticity in their ability to differentiate between herbivorous insect species and subtly adjust their defense responses to target distinct pests. One key mechanism used by plants to recognize herbivorous caterpillars is elicitors present in their oral secretions; however, these elicitors not only cause the induction of plant defenses but recent evidence suggests that they may also suppress plant responses. The absence of "expected changes" in induced defense responses of insect-infested plants has been attributed to hydrogen peroxide produced by caterpillar salivary glucose oxidase (GOX). Activity of this enzyme is variable among caterpillar species; it was detected in two generalist caterpillars, the beet armyworm (*Spodoptera exigua*) and the bertha armyworm (*Mamestra configurata*), but not in other generalist or specialist caterpillar species tested. In the beet armyworm, GOX activity fluctuated over larval development with high activity associated with the salivary glands of fourth instars. Larval salivary GOX activity of the beet armyworm and the bertha armyworm was observed to be significantly higher in caterpillars reared on artificial diet as compared with those reared on *Medicago truncatula* plants. This implies that a factor in the diet is involved in the regulation of caterpillar salivary enzyme activity. Therefore, plant diet may be regulating caterpillar oral elicitors that are involved in the regulation of plant defense responses: our goal is to understand these two processes.

**[Enzymes salivaires de chenille : la « stimulation » d'une réaction]**

Les plantes font montre d'une remarquable plasticité pour distinguer différentes espèces d'insectes herbivores et subtilement ajuster leurs réponses de défense en fonction des différents ravageurs. Les éliciteurs présents dans les sécrétions orales constituent un mécanisme clé utilisé par les plantes pour reconnaître les chenilles herbivores; cependant, ces éliciteurs, non seulement provoquent-ils l'induction des défenses de la plante, mais des preuves récentes suggèrent qu'ils pourraient aussi inhiber les réponses de la plante. L'absence de « changements attendus » en guise de réponses de défense chez des plantes infestées d'insectes a été attribuée au peroxyde d'hydrogène produit par la glucose oxydase (GOX) salivaire de la chenille. L'activité de cette enzyme varie selon l'espèce de chenille; elle fut détectée chez deux espèces de chenilles généralistes, la légionnaire de la betterave (*Spodoptera exigua*) et la légionnaire bertha (*Mamestra configurata*), mais pas chez les autres espèces de chenilles généralistes ou spécialistes testées. Chez la légionnaire de la betterave, l'activité de la GOX fluctua pendant le développement larvaire avec une forte activité associée aux glandes salivaires du 4<sup>e</sup> stade larvaire. On observa que l'activité salivaire de la GOX chez les larves de la légionnaire de la betterave et chez la légionnaire bertha était significativement plus élevée chez les chenilles élevées sur un milieu nutritif artificiel que chez celles élevées sur des *Medicago truncatula*. Ceci suppose qu'un facteur du régime alimentaire est impliqué dans la régulation de l'activité des enzymes salivaires des chenilles. Par conséquent, le régime alimentaire végétal pourrait réguler les éliciteurs oraux de la chenille qui seraient impliqués dans la régulation des réponses de défense de la plante : notre but est de comprendre ces deux processus.

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## INTRODUCTION

Plant responses to caterpillar herbivory have traditionally been viewed as an extension of the wound response. Now, it is clear that although there are shared components with mechanical damage, insect herbivory elicits distinct responses in plants (Kessler and Baldwin 2002). For example, transcript patterns in mechanically damaged *Arabidopsis* plants were more similar to plants subject to drought stress than to herbivory by caterpillars of the cabbage butterfly, *Pieris rapae* (Reymond *et al.* 2000). In fact, plants are often able to differentiate between Lepidopteran species and larval developmental stages and activate specific responses against distinct pests (DeMoraes *et al.* 1998; Stotz *et al.* 2000; Takabayashi *et al.* 1995). Therefore, the plant must be able to recognise the caterpillar and adjust its defensive pathways to target the herbivore.

### Plant recognition of the caterpillar herbivore

Plants recognize caterpillar herbivores by integrating informative signals, including footsteps on leaf tissue (Bown *et al.* 2002; Kessler and Baldwin 2002). However, one of the best-studied mechanisms of herbivore detection by the plant is through elicitors present in insect oral secretions (Kessler and Baldwin 2002). As caterpillar herbivores initiate their feeding process, saliva is secreted from the labial and mandibular salivary glands onto plant tissue that has been macerated by the insect mandibles, assisted by the maxillae laciniae (Elzing 1987). These secretions mix and transport the ground plant material into the oral cavity. Therefore, there is direct contact between caterpillar oral secretions and undamaged plant tissues. Recognition compounds or elicitors present in these caterpillar oral secretions include volicitin, *N*-(17-hydroxylinolenoyl)-L-glutamine, a fatty acid-amino acid conjugate, and the salivary enzyme glucose oxidase (GOX) (Alborn *et al.* 1997, 2000; Musser *et al.* 2002). Treatment of corn seedlings with volicitin induces the biosynthesis and release of volatile compounds with a similar profile to those released by the plant in response to actual caterpillar herbivory (Alborn *et al.* 1997). These terpenoid and indole volatiles are detected by and attract predators or female parasitoids of the herbivorous caterpillar (Kessler and Baldwin 2001; Degenhardt *et al.* 2003; Dicke *et al.* 2003).

### Glucose oxidase

Elicitors not only induce plant responses, but recent evidence suggests that they may also suppress defensive pathways (Musser *et al.* 2002; Bede and Korth, unpublished data). GOX, the predominant enzyme in the labial salivary glands of caterpillars of the corn earworm, *Helicoverpa zea* (Eichenseer *et al.* 1999), catalyzes the oxidation of glucose forming gluconate and hydrogen peroxide ( $H_2O_2$ ). It has been proposed by Eichenseer and coworkers (1999) that the  $H_2O_2$  produced in this reaction may function as an oxygen scavenger to maintain a relatively anaerobic midgut environment reducing the reactivity of certain plant secondary compounds, as an antimicrobial agent against insect pathogens present on the leaf surface or as a signalling molecule which can modify plant responses.

At low concentrations,  $H_2O_2$  acts as a signalling molecule in the plant (Vandenabeele *et al.* 2003). During incompatible plant-pathogen interactions, endogenous  $H_2O_2$  levels increase and have been implicated in localized cell death during the hypersensitive response of some plant species against invading pathogens (De Gara *et al.* 2003). Recent studies on induced plant defences at the molecular and biochemical levels suggest that the  $H_2O_2$  produced by caterpillar salivary GOX also acts as a signalling molecule in the plant and interferes with "normal" plant antiherbivore defence responses (Musser *et al.* 2002; Bede and Korth, unpublished data). In tobacco plants, the toxic secondary metabolite nicotine is synthesized in response to mechanical damage or chewing herbivory (McCloud and Baldwin 1997). In plants subject to herbivory by caterpillars of the corn earworm, *H. zea*, with physically impaired salivary secretions, this induction of nicotine biosynthesis is higher than in plants exposed to caterpillars with normal salivary secretions (Musser *et al.* 2002). This suppression of induced nicotine biosynthesis can be mimicked by applying GOX to mechanically wounded leaf tissue (Musser *et al.* 2002). This implies that the  $H_2O_2$  produced by salivary GOX acts as a signalling molecule that interferes with plant responses to wounding and chewing herbivory through "cross-talk" with other plant pathways (Felton and Korth 2000).

In a subsequent study, the effect of labial salivary secretions on transcript expression patterns of key regulatory enzymes in the terpenoid pathways was investigated (Bede and Korth, unpublished data). In response to herbivory, volatile terpenoid compounds are synthesized and released by many plant species (Degenhardt *et al.* 2003; Dicke *et al.* 2003). In plants, terpenoids are synthesized through two distinct biosynthetic pathways: the cytosolic mevalonate pathway and the plastid-associated 2C-methyl D-erythritol 4-phosphate (MEP) pathway (Eisenreich *et al.* 2001; Lichtenthaler 1999; Rohmer 1999). Not only are these pathways biochemically unique, but they appear to be dichotomous; monoterpenes, sesquiterpenes and triterpenes are synthesized through the mevalonate pathway and diterpenes and tetraterpenes are synthesized through the MEP pathway (Eisenreich *et al.* 2001; Jux *et al.* 2001). However, there is limited interchange of terpenoid biosynthetic precursors between these two pathways (Bick and Lange 2003). This flexibility may be important in times of stress, such as in response to caterpillar herbivory, to increase terpenoid flux (Jux *et al.* 2001; Piel *et al.* 1998). Transcript expression of *dxr*, the gene encoding 1-deoxy-D-xylulose 5-phosphate reductoisomerase that catalyzes the first committed step in the MEP pathway, was lower in leaves fed upon by caterpillars of the beet armyworm, *Spodoptera exigua*, compared with control plants (Bede and Korth, unpublished data). Lower gene expression levels could be emulated by wounding the leaves and applying either GOX or  $H_2O_2$ . Again, these results suggest that  $H_2O_2$  produced by caterpillar salivary GOX modifies plant defence responses.

### Caterpillar salivary reductoisomerases

Since  $H_2O_2$  influences plant defence responses to caterpillar herbivory, salivary gland homogenates of

fourth larval instars of the beet armyworm, *S. exigua*, were assayed for enzymes involved in the production or degradation of  $H_2O_2$  or other reactive oxygen species signalling molecules. Two caterpillar salivary reductoisomerases, ascorbate peroxidase, which catalyzes the oxidation of vitamin C and concomitant reduction of  $H_2O_2$ , and GOX have been characterized in caterpillar salivary glands of the corn earworm, *H. zea* (Eichenseer *et al.* 1999; Mathews *et al.* 1997). In other insect orders, catalase, an enzyme that catalyzes the degradation of  $H_2O_2$ , and superoxide dismutase (SOD), which catalyzes the removal of highly cytotoxic superoxide radicals producing less toxic hydrogen peroxide, have also been identified (Madhusudhan *et al.* 1994; Ni *et al.* 2000). Using in-gel enzyme assays, GOX, peroxidase, catalase and SOD activity was assessed in salivary gland homogenates of *S. exigua* (Manchenko 2003). Enzymatic activity was evaluated over a neutral to basic pH range since the oral secretions of caterpillars fed artificial diet are neutral in comparison with those of caterpillars reared on plants that were alkaline (~ pH 9) (Bede, personal observation). In these enzyme assays, only GOX activity was detected in the labial salivary gland homogenates of *S. exigua* caterpillars.

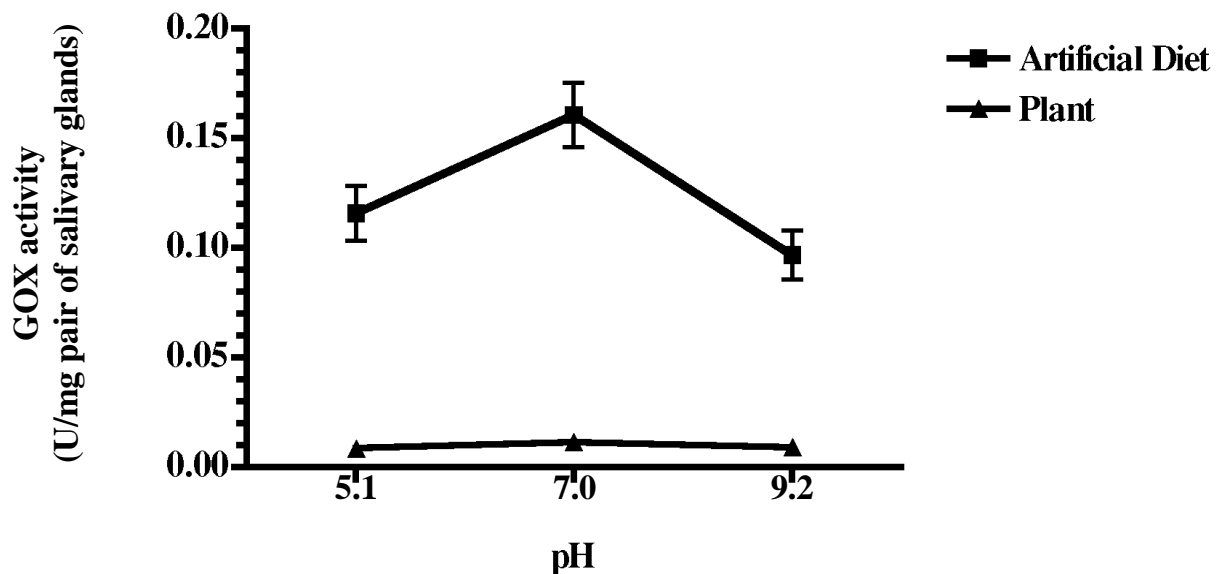
GOX has been identified in the salivary secretions of the generalist Lepidopteran pests *H. zea* and *S. exigua* (Eichenseer *et al.* 1999). Is salivary GOX a common strategy of generalist Lepidopteran species to circumvent plant defences or is this mechanism also used by specialist caterpillar herbivores? The presence of salivary GOX was variable in the Lepidopteran species assayed. Activity was detected in labial salivary gland homogenates of the penultimate larval instars of the beet armyworm, *S. exigua*, and

in the Bertha armyworm, *Mamestra configurata*, but was not detected in other caterpillar species, including other Noctuids such as the true armyworm, *Pseudaletia unipuncta*, or the specialist alfalfa butterfly, *Colias eurytheme*.

Diet had a strong affect on caterpillar salivary gland GOX activity (Fig. 1); the activity of fourth instar *S. exigua* caterpillars reared on artificial diet was over ten times higher than those fed plants. When caterpillars reared on plant diet were transferred to artificial diet, GOX activity increased in proportion to the amount of time spent feeding on the artificial diet (Fig. 2). Caterpillars actively fed on both plant and artificial diet, suggesting that observed differences in GOX activity reflected nutritive status rather than volumetric regulation of feeding.

### Model and future directions

Numerous studies have shown that elicitors, such as  $H_2O_2$  produced by salivary GOX, present in caterpillar oral secretions influence plant defence responses, either by inducing or suppressing biosynthetic pathways (Kessler and Baldwin 2002; Musser *et al.* 2002; Bede and Korth, unpublished data). It is also recognized that diet strongly affects the profile of insect salivary enzymes, including GOX (Hickey *et al.* 1994; Mathews *et al.* 1997). In the fruitfly, *Drosophila melanogaster*, glucose represses transcript expression of the salivary enzyme  $\alpha$ -amylase, thereby regulating its activity (Hickey *et al.* 1994). Whether or not a similar mechanism operates in Lepidopterans is, at present, unknown. Therefore, we present a simplistic model whereby caterpillar elicitors affect plant defence responses and plant nutritional quality affects the salivary enzyme profile (Fig. 3).



**Figure 1. Labial salivary glucose oxidase activity of *Spodoptera exigua* caterpillars: Comparison between caterpillars reared on plants or artificial diet.** Labial salivary glands were dissected from fourth instar *Spodoptera exigua* caterpillars that had been reared on either *Medicago truncatula* plants or a pinto-bean based artificial diet (Bio-Serv). Salivary glands were homogenized in the presence of protease inhibitor (Sigma) and glucose oxidase activity was measured spectrophotometrically (460 nm) using a horseradish peroxidase-coupled reaction with o-dianisidine and glucose as substrates.

Lanes: 1

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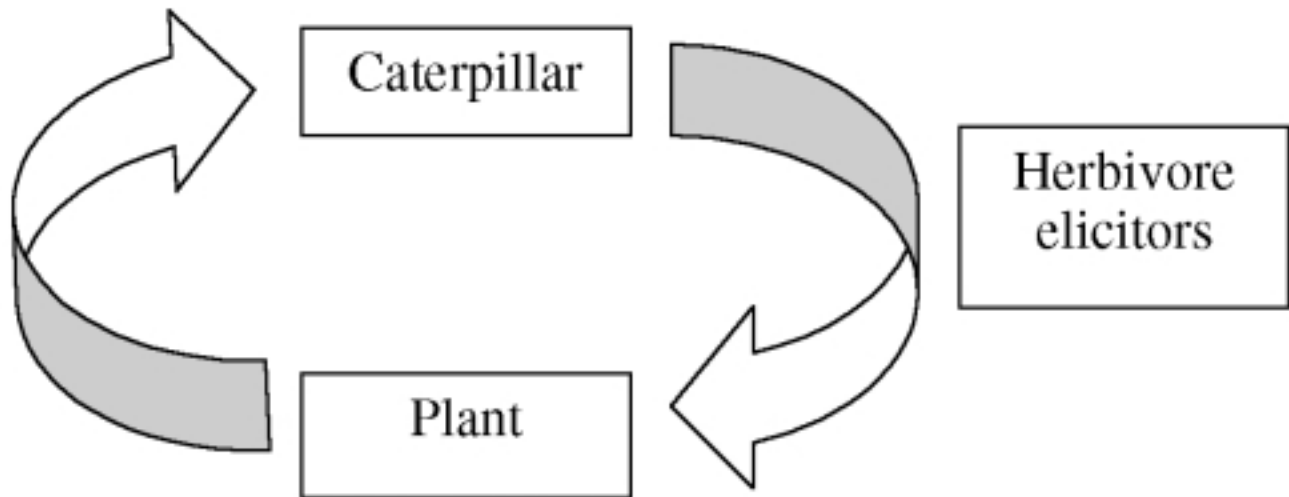
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**Figure 2. *Spodoptera exigua* salivary gland glucose oxidase activity: Feeding time course.** Caterpillars were reared on *Medicago truncatula* plants and transferred to a pinto-based artificial diet (Bio-Serv) for 1 h, 3 h or 12 h. Labial salivary glands of 4<sup>th</sup> instar caterpillars were dissected, homogenized and separated by electrophoresis on native gradient polyacrylamide gels (4-15%). Glucose oxidase (GOX) activity was determined by a peroxidase-coupled assay with glucose and o-dianisidine as the substrates. Lanes: 1) Fungal GOX positive control. 2) Boiled homogenate; time "0". 3) Salivary homogenate from caterpillars reared on plants (time 0). 4) Boiled homogenate; time "1 h". 5) Salivary homogenate from plant-reared caterpillars which had been transferred artificial diet for 1 h. 6) Boiled homogenate; time "3 h". 7) Salivary homogenate from plant-reared caterpillars which had been transferred artificial diet for 3 h. 8) Boiled homogenate; time "12 h". 9) Salivary homogenate from plant-reared caterpillars which had been transferred artificial diet for 12 h.



**Figure 3. Model of plant-caterpillar interactions.** Caterpillar salivary enzymes, such as glucose oxidase, modify plant defence responses. Plant diet affects the profile of the insect salivary enzymes.

Future studies will continue to investigate caterpillar salivary responses to plant food quality and examine how putative salivary elicitors manipulate plant defensive pathways. Once this is elucidated, we can investigate the effects of biotic and abiotic interactions, such as legume-Rhizobium associations, on plant responses to caterpillar herbivory in order to develop a more sophisticated model of plant interactions. Through a comprehensive understanding of these associations, we will generate a strong basis for developing pest control strategies that increase plant productivity through the enhancement of the plant's natural defence mechanisms and/or the identification of potential pesticide targets.

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