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OVEREXPRESSION OF GLUTAMATE DECARBOXYLASE IN TRANSGENIC TOBACCO PLANTS DETERS FEEDING BY PHYTOPHAGOUS INSECT LARVAE

KENNAWAY B. MACGREGOR,[1] BARRY J. SHELP,[2] SRIYANI PEIRIS,[2] and ALAN W. BOWN[1]*

[1]*Department of Biological Sciences*
Brock University
St. Catharines, Ontario, Canada L2S3A1

[2]*Department of Plant Agriculture*
Bovey Building
University of Guelph
Guelph, Ontario, Canada N1G 2W1

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Abstract—Gamma-aminobutyrate (GABA) is a ubiquitous four-carbon, nonprotein amino acid synthesized by glutamate decarboxylase. Previous research suggests that the endogenous synthesis of GABA, a naturally occurring inhibitory neurotransmitter at neuromuscular junctions, serves as a plant resistance mechanism against invertebrate pests. In this study, two homozygous transgenic tobacco lines constitutively overexpressing a single copy of a full-length chimeric glutamate decarboxylase cDNA and possessing enhanced capacity for GABA accumulation (*GAD* plants), a homozygous transgenic line lacking the gene insert, and wild-type tobacco were employed. Tobacco budworm larvae were presented with plantattached wild type and transgenic leaves for 4 hr in a feeding preference study. Larvae consumed six to twelve times more leaf tissue from wild-type plants than from GAD plants. These results suggest that leaf GABA accumulation, which is known to occur in response to insect larval walking and feeding, represents a rapidly deployed localresistance mechanism.

Key Words—Gamma-aminobutyrate, GABA, glutamate decarboxylase, phytophagous, plant resistance, insect larvae, transgenic plants, invertebrate pests.

* To whom correspondence should be addressed. E-mail: bown@spartan.ac.brocku.ca

INTRODUCTION

GABA is a four-carbon, non-protein amino acid found in virtually all prokaryotic and eukaryotic organisms. GABA is synthesized via the alpha-decarboxylation of glutamate in an irreversible reaction catalyzed by the cytosolic enzyme glutamate decarboxylase (GAD; EC 4.1.1.15). It is metabolized in the mitochondrion to succinic semialdehyde by GABA transaminase (EC 2.6.11.9), and then to succinate by succinate semialdehyde dehydrogenase (EC 1.2.1.16). These three enzymecatalyzed reactions constitute the GABA shunt pathway of glutamate metabolism (Bown and Shelp, 1997). In plants, diverse abiotic stresses including mechanical stimulation, mechanical damage, cold shock, heat shock, hypoxia, cytosolic acidification, and water stress stimulate rapid and large GABA accumulation (Bown and Shelp, 1997 and references therein).

A variety of roles for stress-induced GABA accumulation has been suggested (Bown and Shelp, 1997). In invertebrates, GABA is an inhibitory neuromuscular transmitter acting at GABA-gated Cl^- channels. GABA accumulation as a plant resistance mechanism against phytophagous insect larvae was first suggested when mechanical stimulation or damage of leaf tissue was shown to result in rapid GABA synthesis (Wallace et al., 1984). In insect larvae, neuromuscular junctions are not protected by a covering of glial cells, and injection of physiological concentrations of neurotransmitters into the hemolymph of *Lucilia sericata* larvae causes reversible paralysis (Irving et al., 1979). Thus, larval neuromuscular junctions are exposed to ingested GABA if it is absorbed into the hemolymph. Many commercial insecticides are agonists or antagonists of the GABA-gated Cl^- current and disrupt normal neuromuscular activity (Casida, 1993; Hosie et al., 1997). High levels of ingested GABA may have a similar disruptive effect. Significant GABA accumulation in tobacco and soybean leaves occurs within 10 min in response to insect larval crawling (Bown et al., 2002).

Recently, McLean et al. (2003) demonstrated that constitutive transgenic expression of GAD in tobacco confers resistance against the northern root-knot nematode, probably via a GABA-based mechanism. In this paper, we used two of the transgenic lines derived in that study to demonstrate that GAD overexpression also confers resistance against tobacco budworm larvae. We suggest that GABA accumulation in responseto insect larval crawling or herbivory deters feeding.

METHODS AND MATERIALS

Two phenotypically normal, independently transformed, homozygous transgenic lines of tobacco (*Nicotiana tabacum* L. cv. Delgold) overexpressing a single copy of a full-length, chimeric native GAD (designated as *GAD* 2 and *GAD* 5 plants), and a "no insert" homozygous transgenic line expressing only the empty vector were employed in this study (McLean et al., 2003). For assay of GAD

activity, seeds were germinated and grown under greenhouse conditions (McLean et al., 2003). At four weeks of age, the leaves were removed from each plant ($N = 3$) and crude activity in leaf extracts was assayed at pH 5.8 after desalting on a G-25 Sephadex column, as described previously (Serraj et al., 1998).

For feeding preference trials, tobacco seeds were germinated and grown, leaf GABA was determined, and tobacco budworm (*Heliothis virescens* Fabricius) egg masses were reared according to Bown et al. (2002). Plants were used at the seven-leaf stage, with leaf number five being placed within the feeding chamber. Fourth and fifth instars weighing 140–150 mg each were employed. One tobacco budworm larva was placed in a feeding chamber containing two leaves, one attached to a wildtype plant and the other attached to a transgenic plant. Thus, each experiment was internally controlled through the use of one larva given equal access to two different leaves. The feeding chamber was constructed from a 100 × 15 mm Petri dish with slots cut on opposite sides to allow the insertion of the two plant-attached leaves. After 4 hr, leaf area consumed was determined using an Analytical Imaging Station (Imaging Research Inc., St. Catharines, ON). The wild type and transgenic lines did not differ significantly when dry weight to leaf area ratios were measured. Thus, leaf are consumed is a valid measure of tissue consumed. For each plant comparison, a minimum of six trials were conducted, and P was calculated using the 1-tailed Wilcoxon Signed-Rank Test.

RESULTS

Assays of total GAD activity demonstrated that the twotransgenic lines, *GAD* 2 and *GAD* 5, have twiceas much activity as either the wild-type or the transgenic line lacking the gene insert (Table 1), confirming that both *GAD*2 and *GAD*5 overexpress GAD.

The mechanical manipulation required to place two plant-attached leaves into a feeding chamber might influence GABA levels and subsequent feeding activity. To investigate this possibility leaves were placed in a feeding chamber without larvae for 2 hr prior to GABA determinations. No significant differences in GABA levels were found between leaves from the chamber and from resting, non-manipulated leaves (data not shown).

TABLE 1. GAD ACTIVITY IN LEAVES OF FOUR-WEEK-OLD WILD-TYPE AND TRANSGENIC TOBACCO LINES

Genotype	GAD activity (μmol · g^{-1} FW · h^{-1}, mean ± s.e.)
Wild-type	24.5 ± 1.3
No-insert control	21.9 ± 2.9
*GAD*2	40.4 ± 5.3
*GAD*5	39.1 ± 1.0

TABLE 2. LEAF AREA CONSUMED DURING FEEDING PREFERENCE ASSAYS BETWEEN WILD-TYPE AND TRANSGENIC TOBACCO LINES

Leaf pair	Leaf tissue consumed (mm^2, mean ± s.e.)	
(1) Wild-type	13.8 ± 3.2	$P = 0.28$
No-insert control	11.6 ± 3.8	
(2) Wild-type	24.9 ± 11.0	$P = 0.047$
*GAD*2	4.4 ± 1.1	
(3) Wild-type	117.3 ± 81.3	$P = 0.013$
*GAD*5	9.2 ± 4.8	

Feeding preference assays were performed in order to determine whether tobacco budworm larvae prefer wild-type tissue to tissue from *GAD* 2 or *GAD* 5 plants. One tobacco budworm larva was placed in a feeding chamber with one plantattached wild-type leaf and one plant-attached transgenic leaf. After 4 hr of feeding, larvae had consumed 6–12 times more leaf tissue from wild-type plants than from *GAD* 2 or *GAD* 5 plants, indicating that they have a significant feeding preference for wild-type tissue (Table 2). Compared with leaf pairs 2 and 3, a lower value of wild type tissue consumed in leaf pair 1 was expected if no deterrencewas associated with the no-insert control leaf. Despite large variations in the total leaf area consumed by individual larvae, the preference for wild-type tissue was consistently observed. Furthermore, comparison of wild-type plants with transgenic plants lacking the gene revealed that this feeding preference was not caused by the transformation process.

DISCUSSION

Several experimental approaches indicate that the transgenic tobacco lines *GAD* 2 and *GAD* 5 overexpress a functional GAD. 1. The resting GABA levels of intact 2-wk-old seedlings grownin the dark were 150–180% of those in the wild-type (McLean et al., 2003). 2. The GABA-producing potential was determined using a freeze-thaw cycle, which stimulates GAD by destroying cellular compartmentation (see below). Leaf GABA levels of 12 wk-old plants were 170–240% of those in the wild-type (McLean et al., 2003). 3. Assays of total activity in GAD2 and GAD5 plants revealed twice as muchGAD compared to wild-type material (Table 1).

In vitro and *in vivo* data demonstrate that GAD activity is stimulated by increases in the cytosolic levels of H^+ or Ca^{2+} (Bown and Shelp, 1997). Mechanical stimulation results in rapid increases in plant cell cytosolic Ca^{2+} levels, and mechanical damage disrupts cellular compartmentation releasing vacuolar stores of Ca^{2+} and H^+ to the cytosol (Bown and Shelp, 1997 and references therein).

Mechanical stimulation and damage both result in GABA accumulation (Wallace et al., 1984), as does larval crawling on leaf tissue (Bown et al., 2002). Similarly, phytophagous activity will disrupt cellular compartmentation and activate GAD.

Converging data now support the hypothesis that mechanically-induced GABA accumulation functions as a resistance mechanism against invertebrate pests. 1. The introduction of GABA levels found in mechanically-stimulated leaf tissue into a synthetic diet, reduces rates of growth, development and survival of the phytophagous larvae of the oblique-banded leaf roller (*Choristoneura rosaceana*) (Ramputh and Bown, 1996). 2. Tobacco budworm larvae crawling on tobacco or soybean leaves stimulate 5- or 10-fold increases in GABA levels within 10 min (Bown et al., 2002). 3. Overexpression of GAD in transgenic tobacco plants confers resistance to the northern root-knot nematode (McLean et al., 2003), as indicated by a significant reduction in egg masses on the root surface. 4. Overexpression of GAD in transgenic tobacco plants reduces feeding of tobacco budworm larvae (Table 2). It is hypothesized that GABA accumulation in response to pest activity represents a rapidly deployed local resistance mechanism. It may function prior to the deployment of local and systemic resistance mechanisms, which are dependant on changes in gene expression of proteinase inhibitors or other defense products (Kessler and Baldwin, 2002).

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