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Spatial association of photosynthesis and chemical defense in *Arabidopsis thaliana* following herbivory by *Trichoplusia ni*

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Because they share common precursors and require significant amounts of energy, photosynthesis and defense against herbivores and pathogens may be inversely related. This relationship was examined in *Arabidopsis thaliana* exposed to herbivory by *Trichoplusia ni* neonates. The spatial pattern of photosynthesis was compared statistically with that of induction of the defense-related cinnamate-4-hydroxylase (C4H) gene across individual leaves exposed to herbivory in transgenic plants harboring a C4H:GUS gene fusion. In portions of the leaf where C4H:GUS expression was upregulated, photosynthesis was depressed, while non-photochemical quenching was increased, suggesting a trade-off between these two processes. However, photosynthetic damage spread further into surrounding areas than the induction of C4H:GUS expression. Photosynthetic depression was observed up to 1 mm from the edges of holes, whereas C4H:GUS induction typically was limited to about 0.5 mm or less from edges. Other mechanisms may be responsible for the spread of photosynthetic damage beyond where C4H-related defense was induced. Alternatively, C4H induction may reflect a subset of defensive responses more limited in their spatial distribution than the downregulation of photosynthesis. The suppression of photosynthesis in remaining leaf tissue represents a 'hidden cost' of herbivore damage.

Introduction

In addition to the removal of portions of leaves, herbivory can reduce photosynthesis in the remaining leaf tissue (Aldea et al. 2006b, Tang et al. 2006, Nabity et al. 2009). One mechanism contributing to this 'indirect' suppression of photosynthesis is the redirection of metabolic resources to defense. Processes related to growth and defenses require similar resources, such as amino acids, nutrients and materials to build new structures (Herms and Mattson 1992). For example, nitrogen is a key nutrient that is needed to make proteins. In *Nicotiana attenuata*, 6% of total nitrogen is allocated to the defense compound nicotine, reducing the availability of nitrogen to make seeds or photosynthetic enzymes (Baldwin et al. 1998). Materials used to build defense structures are not available for growth or

Abbreviations – C4H, Cinnamate-4-hydroxylase; D1 through D5, damage class 1 through 5; F′, F'_m, light-adapted minimum and maximum chlorophyll fluorescence; F_o, F_m, dark-adapted measurements of minimum and maximum chlorophyll fluorescence; F_v/F_m, maximum photosynthetic quantum efficiency; GUS, β-glucuronidase; NPQ, non-photochemical quenching; PSII, Photosystem II; Φ_{PSII}, operating photochemical efficiency of photosystem II.

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reproduction, unless there is turnover (Gómez and Zamora 2002), and mounting an effective defense may therefore cause reductions in growth and fitness (Zavala et al. 2004).

Because photosynthesis provides the building materials and energy that support growth, there may also be a trade-off between photosynthesis and defense. In wild parsnip (*Pastinaca sativa*), the production of defensive furanocoumarins is associated with increased respiration (Zangerl et al. 1997) and decreased photosynthesis in the remaining leaf tissue (Zangerl et al. 2002). In soybean (*Glycine max*), production of reactive oxygen species in response to ozone and soybean mosaic virus infection is inversely correlated with photosynthesis (Aldea et al. 2006b). Infection by the avirulent pathogen *Pseudomonas syringae*, resulting in the hypersensitive response, also downregulates photosynthesis in soybean (Zou et al. 2005). Lower levels of defense also have been related to higher growth rates. A study of seven deciduous, broad-leaf tree saplings revealed that leaves with high photosynthesis generally had lower total phenolics (Matsuki and Koike 2006).

In addition to the potential trade-offs between photosynthesis and defense, the production and deployment of defensive compounds may be toxic. Following mechanical damage, wild parsnip leaves produce greater quantities of furanocoumarins, but have lower levels of sugar (Zangerl and Berenbaum 1998). Furanocoumarin may damage the photosynthetic apparatus, decreasing sugar output. In addition, defensive terpenes are strongly autotoxic and directly inhibit photosynthesis (Gog et al. 2005), and the amount of photosystem II (PSII) antenna pigments decreases in isolated *Asparagus sprengeri* mesophyll cells undergoing the hypersensitive response (Allen et al. 1999).

Jasmonic acid is one of three major 'defense' hormones produced by plants (Spoel and Dong 2008, Howe and Jander 2008, Lopez et al. 2008), and the inverse relationship between defense and growth-related processes is partly controlled by the jasmonate cascade. In plants exposed to herbivory, the jasmonate cascade helps to control the balance between upregulating defense mechanisms and downregulating non-defense-related processes (Baldwin and Preston 1999). Methyl jasmonate increases expression of defense-related genes (Görscehn et al. 1997, Wasternack and Parthier 1997), but decreases expression of some photosynthetic genes including rubisco (Schenk et al. 2000), as well as decreasing total protein content (Weidhase et al. 1987). The action of this hormone may regulate the inverse expression of defense-related genes and photosynthetic genes. Although induction of the jasmonate cascade is costly because it reduces fitness in undamaged plants, it protects plants under attack and allows them to have greater fitness (Baldwin 1998).

Previously, we demonstrated that the nature of the damage inflicted by *Trichoplusia ni* larvae determines the degree of photosynthetic impairment in *Arabidopsis* (Tang et al. 2006). First instar damage causes photosynthetic depression in the remaining leaf tissue near the holes, whereas fourth instars had little effect on photosynthesis. First instar larvae typically make small holes and avoid veins. Induction of defense in areas near the holes may have contributed to the observed decrease in photosynthesis.

To examine the relationship between the induction of defense and the downregulation of photosynthesis, spatially resolved measurements of these processes were made on the same leaf. We hypothesize that the induction of defense-related phenylpropanoid metabolism partly explains decreased photosynthesis in the remaining leaf area. Specifically, the spatial pattern of cinnamate-4-hydroxylase (C4H) gene expression was imaged as a measure of phenylpropanoid metabolism. C4H is the first cytochrome P450 monooxygenase in the phenylpropanoid pathway (Bell-Lelong et al. 1997). Products of this pathway include defense compounds, such as flavonoids, coumarins and lignin (Dixon and Paiva 1995). In *Arabidopsis*, enzymes in the phenylpropanoid pathway may contribute to defense against pathogens (Dong et al. 1991). C4H is constitutively expressed in the veins of undamaged leaves and induced by wounding near the site of mechanical damage in *Arabidopsis* (Bell-Lelong et al. 1997). The C4H gene is also induced by light and fungal elicitors (Russell 1971, Fahrendorf and Dixon 1993, Logemann et al. 1995). Transgenic *Arabidopsis thaliana* plants with a reporter gene for C4H were exposed to herbivory by *T. ni* to compare effects of damage on photosynthesis and defense-related gene expression.

Materials and methods

Wild-type *A. thaliana* ecotype Columbia and transgenic plants carrying a C4H promoter and β-glucuronidase (GUS) reporter gene fusion (C4H:GUS, Bell-Lelong et al. 1997) were grown in a controlled environment chamber at 160 µmol m^{-2} s^{-1} under a 12-h photoperiod at 20°C. Plants were exposed to first instar *T. ni* larvae obtained from a colony maintained by the Entomology Department of the University of Illinois at Urbana-Champaign. *T. ni* is a generalist herbivore that feeds on many crucifers. Larvae were confined to the underside of one leaf per plant in a clip cage (internal diameter = 165 mm^2) and allowed to feed for 24 h. Measurements began 24 h after larvae were removed from plants.

To expose C4H:GUS plants to a low or high level of herbivory, plants received either two or six larvae. Because the amount of herbivory was variable and did not correspond with the number of larvae placed on each leaf, 13 plants were categorized as receiving either low or high levels of herbivory, based on area removed and not by the number of larvae (low: 8.0 ± 2.8 mm^2, high: 17.0 ± 3.7 mm^2). Empty clip cages were placed on C4H:GUS plants as controls. To verify that the blue dye produced by the assay for GUS activity represented C4H gene expression in transgenic plants, wild-type plants that had received a similar level of herbivory (either two or six larvae) were stained for GUS activity as negative controls.

Leaf gas exchange and chlorophyll fluorescence were measured as in Tang et al. (2006). The leaf was placed in a 2×3-cm gas exchange cuvette with a modified cover consisting of anti-reflective coated glass (NT46-103, Edmund Industrial Optics, Barrington, NJ). Rates of dark respiration, net assimilation, transpiration in the dark-adapted state and transpiration in the light-adapted state were measured with an infrared gas analyzer (LI-6400 Photosynthesis System, LI-COR Biosciences, Lincoln, NE) immediately before dark- and light-adapted chlorophyll fluorescence images were collected. All gas exchange measurements were corrected to account for the missing leaf area.

Chlorophyll fluorescence measurements were taken with a custom-built imager described in Zangerl et al. (2002) and Tang et al. (2006). Illumination of the leaf surface was provided by a combination of 1200 red and blue LEDs that delivered measuring and saturating pulses and actinic light. Fluorescence yield was quantified during short-duration (<100 μs) pulses of blue light. Fluorescence images were captured by a progressive scan charge-coupled camera (659×494, 8-bit, 10 Hz; JAI, Laguna Hills, CA). Dark-adapted measurements of minimum and maximum chlorophyll fluorescence (F_o, F_m) were taken to obtain the maximum photosynthetic quantum efficiency [$F_v/F_m = (F_m - F_o)/F_m$]. Leaves were light-adapted at an irradiance of 150 μ*M* m^{-2} s^{-1}for 45 min, and then minimum and maximum chlorophyll fluorescence (F', F'_m) were measured to obtain the operating photochemical efficiency of PSII [$\Phi_{PSII} = (F'_m - F')/F'_m$]. Dark- and light-adapted measurements of maximum chlorophyll fluorescence were used to calculate non-photochemical quenching ($NPQ = F_m/F'_m - 1$; Oxborough 2006).

Immediately after measuring photosynthesis, leaves were stained for GUS activity to detect the spatial pattern of C4H gene induction following herbivory. After imaging leaves with a digital camera (Coolpix 950, Nikon, Long Island, NY), they were vacuum-infiltrated with GUS stain [100 m*M* NaH_2PO_4/Na_2HPO_4 (pH = 7.5), 0.5 m*M* K ferrocyanide, 0.5 m*M* K ferricyanide, 0.1% Triton X-100, 10 m*M* EDTA, 1 m*M* X-Gluc] and incubated at 37°C for 3–5 h. Chlorophyll was removed from stained samples by immersing them in 70% (v/v) ethanol at 37°C for 3 days, with daily changes of 70% ethanol. Leaves were flattened by placing them on 1% agarose plates and imaged above a light box with a digital camera.

The effects of herbivory on leaf gas exchange were compared by one-way ANOVA (SAS 9.1, SAS Institute, Inc., Cary, NC). Rates of dark respiration, assimilation, transpiration in the dark, and transpiration in the light were compared among undamaged control leaves and leaves exposed to low or high herbivory.

To determine whether areas of suppressed photosynthesis corresponded to the induction of C4H gene expression, images were subjected to geographic image analysis (ERDAS IMAGINE 9.0, Leica Geosystems GIS & Mapping LLC, Atlanta, GA) according to Aldea et al. (2006a). Images of GUS-stained leaves were registered to their corresponding dark-adapted minimal fluorescence images to place the chlorophyll fluorescence and GUS images on the same scale and orientation. Then, images of GUS activity were placed in two-layer stacks with either images of F_v/F_m, Φ_{PSII} or NPQ. The stacks were analyzed to compare GUS activity with each photosynthetic parameter for every point on the leaf. Because some parts of a leaf may respond to damage more than other parts (e.g. those located near holes), leaf area was categorized into groups based on the similarity of their photosynthetic and defense responses. The Unsupervised Classification Tool, a multivariate clustering technique based on an iterative self-organizing algorithm (ISODATA, ERDAS IMAGINE 9.0), was used to group pixels into 10 classes based on the Euclidean distance of corresponding pixel intensities in the two images of each stack. Pixels within a class were most similar to each other within one image and most different between the two images of the stack. Thus, a class represents leaf area in which there is coordination between the photosynthetic and C4H expression response that is distinct from the other parts of the leaf (i.e. the other classes). This analysis produced a signature file that was used with the Supervised Classification Tool to make a color-coded map of the location of classes (Bayesian Maximum Likelihood classification).

The 10 groupings were categorized as a damage class or part of the undamaged class according to their proximity to the holes and their level of photosynthesis. Classes that corresponded to regions closely associated with holes and depressed in photosynthesis were considered damage classes and numbered according

to their proximity to the edge of the hole. Damage class 1 (D1) was closest to the edge, while damage class 5 (D5) was furthest. One or two of the classes were closely associated with veins, because constitutive expression of the C4H gene in veins causes them to stain blue. Classes closely associated with veins but not holes were excluded from analysis. The remaining classes not associated with veins or holes typically had diffuse patterns that were indistinct from one another. They also had higher values of F_v/F_m and Φ_{PSII} and lower values of NPQ than the damage classes (see section on Results). They were grouped together into one class and considered undamaged. The least square means of F_v/F_m, Φ_{PSII} or NPQ values of each damage class and undamaged area were quantified and compared by two-way ANOVA (SAS 9.1, SAS Institute, Inc.). Level of herbivory (low or high), damage class and the interaction between level of herbivory and damage class were fixed effects in the model.

To determine whether regions of suppressed photosynthesis corresponded with the induction of defense, photosynthesis in defense-induced areas of damaged leaves was compared with undamaged areas. A defense-induced area was defined as a damage class with an intensity of GUS activity staining that was at least 10% greater than the undamaged area. This value was chosen because a 10% difference was greater than typical variations in GUS staining between damaged and control areas. Mean F_v/F_m, Φ_{PSII} and NPQ values of each defense-induced area were compared with undamaged areas using a t-test (SAS 9.1, SAS Institute, Inc.).

NPQ was measured to further explore the mechanisms contributing to reduced photosynthesis following herbivory. Because NPQ typically increases with the loss of sinks for ATP and NADPH associated with low intercellular CO_2, an increase in NPQ often accompanies a reduction in stomatal conductance associated with water stress (Omasa and Takayama 2003, Souza et al. 2004). NPQ values of damage classes that were not classified as defense-induced were compared with undamaged areas using a t-test (SAS 9.1, SAS Institute, Inc.).

The spread of photosynthetic damage was quantified by measuring the width of the regions surrounding holes that represented damage classes. Two holes from each of the 13 leaves per level of herbivory were randomly selected for analysis. The width of each damage class was measured at four angles randomly chosen from eight directions (0°, 45°, 90°, 135°, 180°, 225°, 270° and 315°). The width of each damage class was compared by two-way ANOVA (SAS 9.1, SAS Institute, Inc.). Level of herbivory (low or high), damage class and the interaction between level of herbivory and damage class were fixed effects in the model.

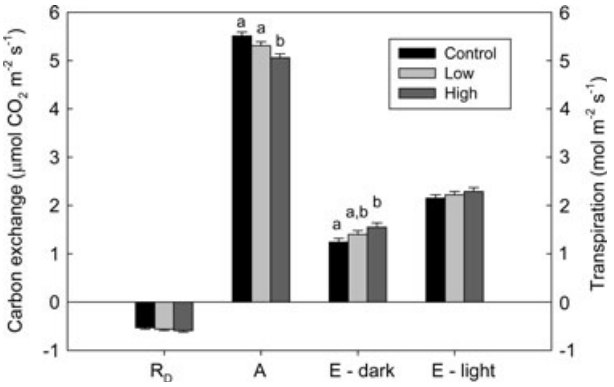

Fig. 1. Effects of herbivory on leaf gas exchange rates. Rates of dark respiration (R_D), assimilation (A), transpiration in the dark (E – dark) and transpiration in the light (E – light) are shown for leaves that were undamaged or exposed to low or high herbivory. Bars represent least square means and error bars are ± SE. There was a significant effect of herbivory on assimilation and transpiration in the dark ($P \leqslant 0.05$). Significant differences between levels of herbivory are indicated with different letters ($P \leqslant 0.05$). Mean ± SD area removed at low herbivory was 8.0 ± 2.8 mm^2 and that removed at high herbivory was 17.0 ± 3.7 mm^2.

Results

Leaf gas exchange measurements, corrected for missing leaf area, revealed that the high level of herbivory caused more damage than the low level (Fig. 1). Net carbon exchange was significantly lower for leaves exposed to high level of herbivory (8% lower relative to control) than those exposed to either low levels of herbivory or the no-herbivory control. Herbivory caused a significant increase in transpiration in the dark ($P \leqslant 0.05$), but not on dark respiration or transpiration in the light. The transpiration rate was 25% greater for leaves exposed to a high level of herbivory than undamaged leaves.

Expression of the C4H:GUS following herbivory was localized to the perimeter of holes (Fig. 2). As expected, the negative control of damaged wild-type plants did not stain blue (Fig. 2A). Undamaged transgenic plants harboring C4H:GUS showed GUS activity staining only at the veins (Fig. 2B), indicating constitutive C4H expression at the veins. In contrast, damaged transgenic plants showed GUS activity staining at the perimeter of each hole as well as within the veins (Fig. 2C). GUS activity was closely associated with the two damage classes closest to the perimeter of holes.

Herbivory caused varying degrees of photosynthetic damage depending on its extent. Overlay of a chlorophyll fluorescence image that reflects PSII activity and a corresponding image of GUS staining (Fig. 3), followed by statistical analysis, revealed four or five damage classes around each hole – regions where pixel intensity shared common traits between the two

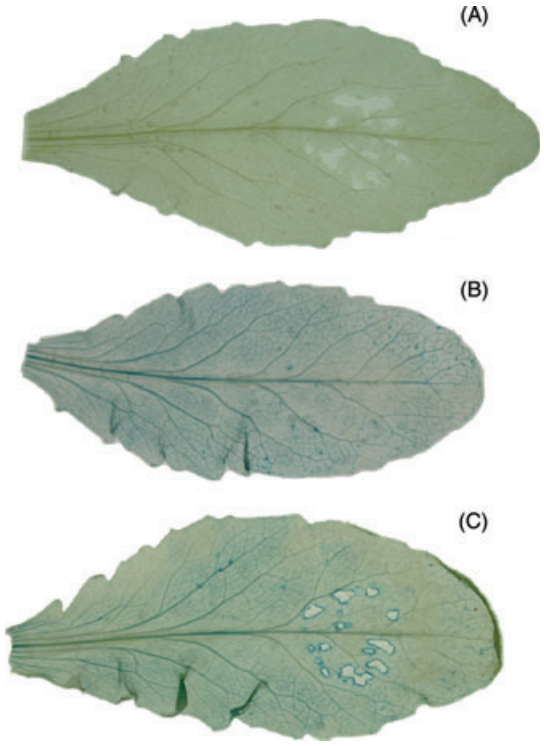

Fig. 2. Images of GUS-stained leaves illustrating the induction of the gene coding for C4H, a gateway defense protein in phenylpropanoid metabolism. (A) Wild-type, control leaf exposed to herbivory did not stain blue. (B) Undamaged transgenic leaf with C4H:GUS fusion stained only along the veins. (C) Damaged transgenic leaf with C4H:GUS fusion stained at the veins and at the perimeter of holes.

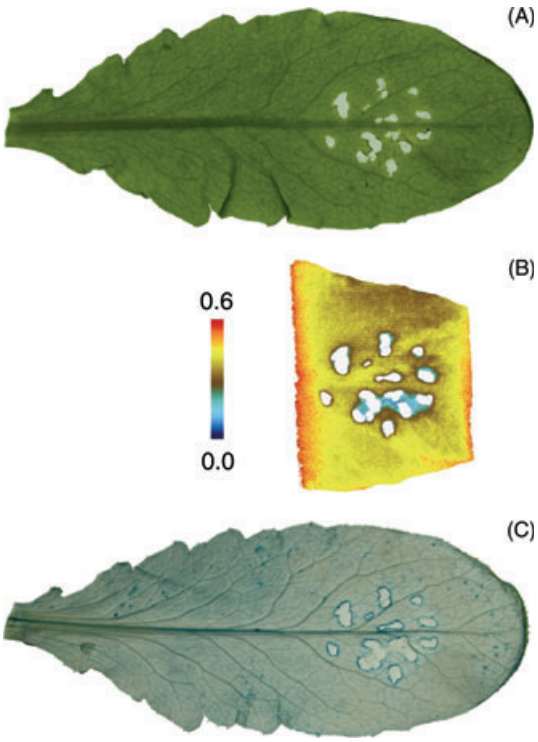

Fig. 3. Images of a leaf exposed to a high level of herbivory. (A) Visible light image of the leaf before GUS staining. (B) False color Φ_{PSII} image in which the color of each pixel represents the level of Φ_{PSII} at that location. (C) Visible light image of leaf after GUS staining. Images (B) and (C) were overlaid to form a stack and statistically analyzed with geographical imaging software to determine damage classes, as defined in section on Materials and methods. A damage class is a group of pixels that are most similar to each other within one image and most different between the two images of the stack (in this example, GUS staining and Φ_{PSII}).

images in a stack, as determined by the automated clustering algorithm. Photosynthetic damage was most severe at the edge of holes but diminished with distance from the holes (Fig. 4), a pattern that inversely correlates with that of C4H:GUS induction. The maximum photochemical efficiency of PSII (F_v/F_m) and the operating photochemical efficiency of PSII (Φ_{PSII}) had similar patterns of photosynthetic depression following herbivory. Values of F_v/F_m and Φ_{PSII} were lowest near the holes and increased further away from the damage. NPQ was greatest near the edge of holes, especially in regions between holes (Fig. 4). A large percentage of the damage classes categorized as defense-induced in the F_v/F_m, Φ_{PSII} or NPQ image stacks were either damage classes 1 or 2 (90, 74 and 68%, respectively).

The spread of photosynthetic damage was affected by the level of herbivory. There was a significant effect of herbivory on the halo widths of Φ_{PSII} and NPQ damage classes, but not on F_v/F_m (Table 1). The halo widths of Φ_{PSII} and NPQ damage classes were, respectively, 1.6-fold and 2-fold greater at low herbivory than at high herbivory (Table 2). The interaction between herbivory and damage class was not significant for the halo widths of either Φ_{PSII} or NPQ damage classes. Although halo width generally increased with damage class (from D1 to D5) by definition, there were exceptions caused by variation between holes within a leaf and the number of damage classes in different leaves. In addition, NPQ values of damage classes under high herbivory were not significantly different from one another.

The level of herbivory did not affect the pattern of variation in mean F_v/F_m, Φ_{PSII} or NPQ among damage classes (e.g. D1, D2) within a treatment level (i.e. low or high herbivory, Table 3). Among the damage classes produced under low herbivory, F_v/F_m of the damage class located closest to the edge of holes (D1) was significantly lower than the damage classes that were further away (D2 through D5). In addition, there were no significant differences among the mean F_v/F_m values of damage classes D2 through D5, indicating that physical damage to the photosynthetic apparatus occurred within

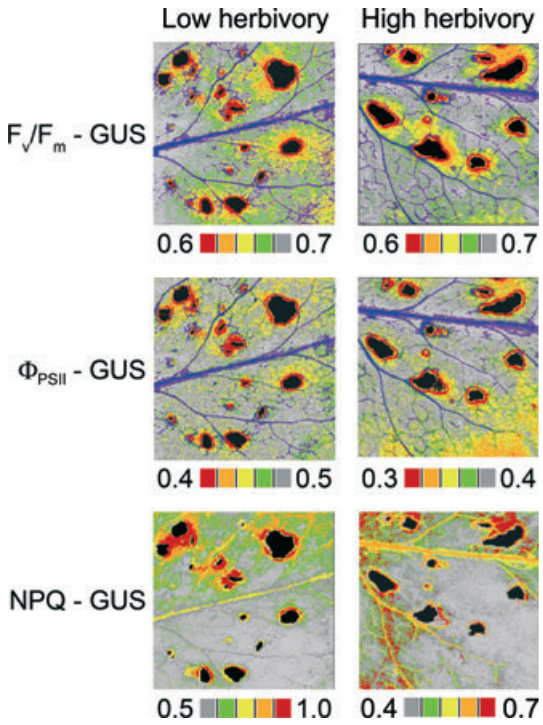

Fig. 4. False color images of the location of damage classes surrounding holes in a leaf exposed to low or high levels of herbivory. A damage class is a group of pixels that are most similar to each other within one image and most different between the two images of the stack (GUS staining and either F_v/F_m, Φ_{PSII} or NPQ). The false color scale bars indicate the mean value of F_v/F_m, Φ_{PSII} or NPQ for each damage class. The veins shown in blue and purple were classes that were excluded from analysis because their high level of GUS staining was not related to herbivory. Mean ±SD area removed at low herbivory was 8.0 ± 2.8 mm^2 and that removed at high herbivory was 17.0 ± 3.7 mm^2.

about 0.3 mm from the edge of holes (Tables 2 and 3). The same pattern was observed for F_v/F_m damage classes produced under high herbivory. Values of Φ_{PSII} also were lowest adjacent to the hole but gradually increased with distance from the damage for both treatments. There was no significant difference in NPQ among the damage classes (D1 through D4) at both low and high herbivory.

Table 1. Analysis of variance for the width of each damage class from the edge of the holes to the outer limit of each damage class, for leaves exposed to low or high levels of herbivory. A damage class is a group of pixels that are most similar to each other within one image and most different between the two images of the stack (GUS staining and either F_v/F_m, Φ_{PSII} or NPQ). The effects of herbivory and damage class and the interaction between them were tested. The degrees of freedom (df), F test (F) and probability (*P*) values are reported for the ANOVA.

Main effects and interactions	df	F	*P*
F_v/F_m			
Herbivory	1	0.78	0.38
Damage class	4	8.35	<0.01
Herbivory × damage class	4	0.62	0.65
Φ_{PSII}			
Herbivory	1	10.67	<0.01
Damage class	4	10	<0.01
Herbivory × damage class	4	1.45	0.22
NPQ			
Herbivory	1	14.87	<0.01
Damage class	3	2.76	0.05
Herbivory × damage class	3	0.24	0.87

The degree of photosynthetic depression in the area surrounding holes depended on the level of herbivory (Table 3). Within a level of herbivory, the area closest to the edge of the hole (D1) typically had significantly lower F_v/F_m and Φ_{PSII} than the other four damage classes. Herbivory had a significant effect on both F_v/F_m and NPQ (Table 4). Values of F_v/F_m at high herbivory were 5% lower than those at low herbivory ($P = 0.02$). In contrast, NPQ values at high herbivory were 28% higher than those at low herbivory ($P = 0.02$). Values of Φ_{PSII} at high herbivory showed a trend of being lower than those at low herbivory ($P = 0.09$). There were no significant interactions between herbivory level and damage class for F_v/F_m, Φ_{PSII} and NPQ values.

Photosynthesis was depressed in areas of the leaf where defenses were induced (Fig. 5). The maximum photochemical efficiency of PSII (F_v/F_m) and the operating photochemical efficiency of PSII (Φ_{PSII}) were both significantly lower in the C4H-induced areas

Table 2. The least square mean width (mm) of each damage class from the edge of the holes to the outer edge of each damage class for leaves exposed to low or high levels of herbivory (± SE). A damage class is a group of pixels that are most similar to each other within one image and most different between the two images of the stack (GUS staining and either F_v/F_m, Φ_{PSII} or NPQ). Significant differences between damage classes within a treatment (low or high herbivory) are indicated with different letters.

F_v/F_m			Φ_{PSII}			NPQ		
	Low herbivory	High herbivory		Low herbivory	High herbivory		Low herbivory	High herbivory
D1	0.31 ± 0.11^{a}	0.20 ± 0.12^{a}	D1	0.31 ± 0.08^{a}	0.22 ± 0.09^{a}	D1	0.31 ± 0.08^{a}	0.14 ± 0.09^{a}
D2	$0.51 \pm 0.11^{a,c}$	$0.28 \pm 0.12^{a,c}$	D2	0.35 ± 0.08^{a}	0.32 ± 0.09^{a}	D2	0.55 ± 0.09^{b}	0.24 ± 0.09^{a}
D3	$0.68 \pm 0.11^{b,c}$	$0.61 \pm 0.12^{b,c}$	D3	0.65 ± 0.08^{b}	0.40 ± 0.09^{b}	D3	$0.51 \pm 0.09^{a,b}$	0.26 ± 0.09^{a}
D4	0.99 ± 0.13^{b}	0.80 ± 0.12^{b}	D4	0.93 ± 0.09^{c}	0.54 ± 0.09^{c}	D4	0.61 ± 0.10^{b}	0.35 ± 0.10^{a}
D5	$0.54 \pm 0.21^{a,b}$	0.77 ± 0.17^{b}	D5	$0.90 \pm 0.21^{b,c}$	$0.52 \pm 0.15^{b,c}$			

Table 3. Least square mean values for F_v/F_m, Φ_{PSII} or NPQ for each damage class (± SE). A damage class is a group of pixels that are most similar to each other within one image and most different between the two images of the stack (GUS staining and either F_v/F_m, Φ_{PSII} or NPQ). Mean values of F_v/F_m, Φ_{PSII} and NPQ in undamaged areas were 0.69 ± 0.01, 0.45 ± 0.02, and 0.49 ± 0.10, respectively. Significant differences between damage classes within a treatment (low or high herbivory) are indicated with different letters.

F_v/F_m			Φ_{PSII}			NPQ		
	Low herbivory	High herbivory		Low herbivory	High herbivory		Low herbivory	High herbivory
D1	0.58 ± 0.02^a	0.53 ± 0.02^a	D1	0.35 ± 0.01^a	0.30 ± 0.02^a	D1	0.73 ± 0.09^a	0.99 ± 0.10^a
D2	0.64 ± 0.02^b	0.62 ± 0.02^b	D2	$0.39 \pm 0.01^{a,b}$	0.36 ± 0.02^b	D2	0.59 ± 0.10^a	0.83 ± 0.10^a
D3	0.66 ± 0.02^b	0.63 ± 0.02^b	D3	0.42 ± 0.01^b	0.38 ± 0.02^b	D3	0.67 ± 0.10^a	0.77 ± 0.10^a
D4	0.68 ± 0.02^b	0.64 ± 0.02^b	D4	0.43 ± 0.02^b	$0.41 \pm 0.02^{b,c}$	D4	0.59 ± 0.11^a	0.74 ± 0.11^a
D5	0.66 ± 0.03^b	0.66 ± 0.02^b	D5	$0.42 \pm 0.04^{a,b}$	0.45 ± 0.03^c			

compared with the undamaged portion of the leaf (15 and 19% lower, respectively). The reduction in F_v/F_m suggests that herbivory caused physical damage to the photosynthetic apparatus. NPQ was 41% higher in the defended area compared with the undamaged area. Thus, there was greater non-photochemical dissipation of excitation energy in the defended area, because less excitation energy was used to drive photosynthesis.

NPQ was higher in damage classes that were not C4H-induced compared with undamaged areas. The mean NPQ of all non-induced damage classes was 61% higher than that of the undamaged areas (least square mean ± SE: 0.82 ± 0.25 vs 0.51 ± 0.11).

There was greater propagation of photosynthetic damage in the remaining leaf area compared with defense-induction (Fig. 4). Photosynthetic damage was observed up to 1 mm away from the edge of the hole (Table 2). Most damage classes categorized as defense-induced were damage class 1 or 2. These damage classes extended up to about 0.5 mm from the edge of holes. Thus, photosynthetic damage spread twice as far as defense-induction.

Table 4. Analysis of variance for least square mean of F_v/F_m, Φ_{PSII} or NPQ values of each damage class. A damage class is a group of pixels that are most similar to each other within one image and most different between the two images of the stack (GUS staining and either F_v/F_m, Φ_{PSII} or NPQ). The effects of herbivory, damage class and the interaction between them were fixed effects. The degrees of freedom (df), F test (F) and probability (*P*) values are reported for the ANOVA.

Main effects and interactions	df	F	*P*
F_v/F_m			
Herbivory	1	6.15	0.02
Damage class	4	12.72	<0.01
Herbivory × damage class	4	0.29	0.88
Φ_{PSII}			
Herbivory	1	3.1	0.09
Damage class	4	10.65	<0.01
Herbivory × damage class	4	0.66	0.63
NPQ			
Herbivory	1	6.48	0.02
Damage class	3	1.4	0.26
Herbivory × damage class	3	0.3	0.82

Discussion

In addition to removing leaf tissue, chewing damage by *T. ni* larvae reduced the rate of photosynthesis in surrounding leaf tissue up to 3 mm away from severed edges. This indirect reduction in photosynthesis can be quite large and varies with the type of damage (Aldea et al. 2006b). There was a corresponding induction of C4H gene expression, indicating the induction of a defense response; however, photosynthetic damage propagated further into the surrounding area of the leaf than the induction of C4H gene expression. This may mean that defense-induction does not fully explain the reduction in photosynthesis. However, this conclusion assumes that C4H:GUS induction reflects all defensive activities. Other genes or metabolite changes may have reflected a pattern of change more closely matching that

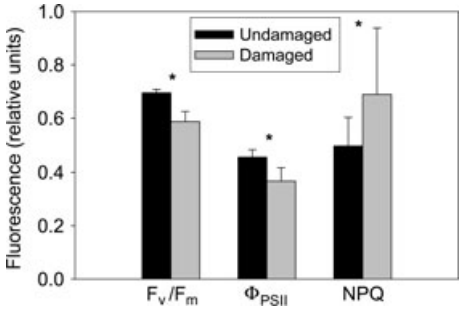

Fig. 5. Mean values of F_v/F_m, Φ_{PSII} and NPQ for damage classes where defense was induced (primarily D1 and D2; 'damaged') compared with undamaged areas (mean ± SE). Damage classes are groups of pixels which share similar traits in GUS staining and either F_v/F_m, Φ_{PSII} or NPQ. A damage class was considered to have induced defenses if the intensity of its GUS staining was 10% greater than the undamaged area. Significant differences between values for damaged and undamaged areas are noted with an '*' ($P \leqslant 0.05$).

of the change in photosynthesis induced by herbivory. In addition, it may also reflect differences in the sensitivities with which changes in GUS activity and photosynthesis can be measured.

Redeployment of resources from photosynthetic processes to defenses that do not involve metabolic flux involving C4H could have contributed to the decrease in photosynthesis. For example, the mevalonic acid and glyceraldehyde/pyruvate pathways produce terpenes (Adam et al. 1998). Glucosinolates are also produced from a biosynthetic pathway separate from phenylpropanoid metabolism (Kliebenstein et al. 2001, reviewed in Halkier and Gershenzon 2006); the hydrolysis products of some glucosinolates protect *Arabidopsis* from herbivory (Mewis et al. 2005, Zhang et al. 2006).

Water stress may also have been responsible for reduced photosynthesis at distance from holes where C4H was not induced (Tang et al. 2006). Small but numerous holes produced by first instar larvae may decrease photosynthesis by isolating remaining tissue from water-conducting vasculature and accelerating water loss from cut edges (Aldea et al. 2005). Localized water stress can in turn reduce photosynthesis by causing stomata to close (reviewed in Hetherington and Woodward 2003) and by decreasing ATP synthase activity (Tezara et al. 1999). NPQ was higher for damage classes, where defense was not induced, compared with undamaged areas. Increased NPQ is associated with low stomatal conductance in plants under drought stress (Omasa and Takayama 2003, Souza et al. 2004), supporting the hypothesis that water stress may have contributed to depressed photosynthesis in areas beyond where C4H was upregulated. One way to assess this idea might be to examine stomatal apertures in damaged leaves as a function of their distance from the sites of herbivory.

The level of herbivory generally had a significant effect on the severity and spread of photosynthetic damage in the remaining leaf area. The number of holes made by larvae was not significantly different between the low and high herbivory treatments (low: 11 ± 5, high: 12 ± 5). Thus, the difference between low and high herbivory appears to be the size of holes. Because the same area was exposed to herbivory, the holes at high herbivory were closer to one another than those of the low herbivory treatment. In addition, low stomatal conductance in dark-adapted, undamaged leaves (0.09 ± 0.02, $n = 7$) suggests that the dark-adapted transpiration rate in damaged leaves was strongly affected by water lost through the cut edge. Though there was no significant difference between high and low levels of herbivory, leaves that experienced a high level of herbivory had 25% greater transpiration rate than undamaged leaves ($P \leqslant 0.05$), suggesting that water stress may have been greater at high herbivory.

Levels of defense and photosynthesis are not always inversely related. It may be more adaptive to protect highly productive leaves with greater levels of defense chemicals (Denno and McClure 1983, Zavala and Baldwin 2004). In *N. sylvestris*, nicotine production and photosynthetic rate are positively correlated (Baldwin and Ohnmeiss 1994). In transgenic *N. tabacum* with decreased expression of transketolase, both the enzyme involved in photosynthesis and the pathway that contributes to phenylpropanoid metabolism, photosynthesis and accumulation of defense compounds decrease (Henkes et al. 2001). Defense may decrease because photosynthesis provides precursors used by defense-related pathways. Defense and photosynthesis may be related to each other under some environmental conditions, but not others. In the oak species *Quercus crispus*, herbivory caused increased production of condensed tannins and decreased photosynthesis at high light, but not at low light (Nabeshima et al. 2001).

The decrease in photosynthesis in portions of the leaf where C4H was induced suggests that there may be a trade-off between defense and photosynthesis. Thus, estimating the cost of defense should include not only allocation of resources to defense, but also loss of capacity to generate energy and materials that support both defense and growth. Because photosynthetic damage spread beyond the area where defense, as measured by C4H:GUS expression, was induced, defense pathways unrelated to phenylpropanoid metabolism, such as glucosinolate biosynthesis, may be among the processes that contributed to photosynthetic damage. Alternatively, other non-defense-related processes may have contributed to the reduction in photosynthesis in these areas including stomatal closure related to localized water stress.

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