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RESEARCH PAPER

The differential effects of herbivory by first and fourth instars of *Trichoplusia ni* (Lepidoptera: Noctuidae) on photosynthesis in *Arabidopsis thaliana*

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Abstract

The effect of different feeding behaviours of 1st and 4th instar *Trichoplusia ni* on photosynthesis of *Arabidopsis thaliana* var. Columbia was characterized using spatially resolved measurements of fluorescence and leaf temperature, as well as leaf gas exchange,. First instars made small holes with a large perimeter-to-area ratio and avoided veins, while 4th instars made large holes with a low perimeter-to-area ratio and consumed veins. Herbivory by 1st instars reduced photosynthesis more strongly in the remaining leaf tissue than that by 4th instars. Photosystem II operating efficiency (Φ_{PSII}) was correlated with the rate of CO_2 exchange, and reductions in Φ_{PSII} in areas around the missing tissues contributed to a 15.6% reduction in CO_2 assimilation on the first day following removal of 1st instars. The corresponding increases in non-photochemical quenching and greater rates of non-stomatal water loss from these regions, as well as the partial reversal of low Φ_{PSII} by increasing the ambient CO_2 concentration, suggests that localized water stress and reduced stomatal conductance contributed to the inhibition of photosynthesis. Damage by 1st but not 4th instars reduced the maximum quantum efficiency of photosystem II photochemistry (F_v/F_m) by 4–8%. While herbivory by both 1st and 4th instars increased dark respiration rates, the rates were too low to have contributed to the observed reductions in CO_2 exchange. The small holes produced by 1st instars may have isolated patches of tissue from the vascular system thereby contributing to localized water stress. Since neither 1st nor 4th instar herbivory had a detectable effect on the expression of the Rubisco small subunit gene, the observed differences cannot be attributed to changes in expression of this gene. The mode of feeding by different instars of *T. ni* determined the photosynthetic response to herbivory, which appeared to be mediated by the level of water stress associated with herbivore damage.

Key words: Fluorescence imaging, green fluorescent protein, photosynthesis, Rubisco small subunit, *Trichoplusia ni*.

Introduction

Herbivory can profoundly affect ecosystems by decreasing photosynthesis and net primary production. Estimates of crop production removed by foliage-feeding insects typically ranges from 5% to 30% (Mattson and Addy, 1975), and insect outbreaks can reduce net primary productivity by

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Abbreviations: Φ_{CO2}, apparent quantum yield of CO_2 fixation (net photosynthesis/incident light); Φ_{PSII}, an estimate of the operating photosynthetic efficiency of photosystem II photochemistry in the light-adapted state; F_o, minimum chlorophyll fluorescence in the dark-adapted state; F_m, maximum chlorophyll fluorescence in the dark-adapted state; F', minimum chlorophyll fluorescence in the light-adapted state; F'_m, maximum chlorophyll fluorescence in the light-adapted state; F_v/F_m, an estimate of maximum quantum efficiency of photosystem II photochemistry in the dark-adapted state; PI, pixel intensity; GFP, green fluorescent protein; LEDs, light-emitting diodes; *NPQ*, non-photochemical quenching; *PFD*, photon flux density; RbcS1a, Rubisco small subunit gene; Rubisco, ribulose-1,5-*bis*phosphate carboxylase/oxygenase.

more than 70% in some terrestrial ecosystems (Cyr and Pace, 1993). The loss of productivity to herbivory traditionally has been estimated as the amount of leaf tissue removed (Ohmart *et al*., 1983; Lowman, 1985). However, this approach may underestimate its impact because it does not consider the potential effect of herbivory on the photosynthetic competence of remaining leaf tissue (Welter, 1989; Zangerl *et al*., 2002; Aldea *et al*., 2005).

Variable responses to herbivory have been observed among different plant–insect combinations. While complete leaf removal (defoliation), particularly of grasses, often causes an increase in the rate of net photosynthesis in the remaining or newly formed leaves, insects that selectively feed on specific tissues without their complete removal often cause a reduction of carbon exchange in the remaining leaf tissue (Welter, 1989). Moreover, the mode of tissue damage modulates its effect on photosynthesis. For example, the removal of leaf tissue from soybean (*Glycine max* L.), by Japanese beetle (*Popillia japonica* Newman), corn earworm (*Helicoverpa zea* Bodie) caterpillars (Aldea *et al*., 2005), cabbage looper (*T. ni* Hübner), and green cloverworm (*Plathypena scabra* F.) (Hammond and Pedigo, 1981; Ostlie and Pedigo, 1984) caused an increase in water loss from damaged tissue, but had minimal effect on net photosynthesis. By contrast, chewing damage to soybean by Mexican bean beetle (*Epilachna varivestis* Mulsant) caused substantial losses of photosynthesis in the remaining leaf tissue (Peterson *et al*., 1998). The scraping and crushing of interveinal leaf tissue by adults and larvae of Mexican bean beetles may exacerbate localized water stress, ultimately causing tissue desiccation.

The indirect suppression of photosynthesis in nearby leaf tissue by insect herbivores is caused by a number of factors, including disruption of water and nutrient transport (Welter, 1989; Sack *et al*., 2004; Nykänen and Koricheva, 2004), fundamental changes in the performance of photosynthetic enzymes (Peterson *et al*., 1998), as well as diverting resources to defence (Zangerl *et al*., 2002). The mechanisms underlying the loss of photosynthesis vary with the type of damage and even its pattern across the leaf surface (Morrison and Reekie, 1995; Mauricio *et al*., 1993).

Chlorophyll fluorescence imaging provides a nondestructive, high-resolution technique to examine the spatial variation in the component processes of photosynthesis following herbivore damage (Baker *et al*., 2001; Genty and Meyer, 1994; Oxborough, 2004; Oxborough and Baker, 1997). The quantum efficiency of photosystem II fluorescence (Φ_{PSII}) is particularly useful in this context as it is related to the rate of carbon fixation (Di Marco *et al*., 1990; Genty *et al*., 1989). Using a fluorescence imaging system, Zangerl *et al*. (2002) found that exposure to herbivory by cabbage loopers (*T. ni*) reduced Φ_{PSII} and the rate of CO_2 uptake of the remaining foliage of wild parsnip (*Pastinaca sativa*), causing a loss of photosynthesis 3-fold greater than from the removal of tissue alone.

The objective of this study was to determine whether different types of damage by a chewing insect differentially affect photosynthesis in adjacent portions of the leaf in *A. thaliana*. The effects of 1st and 4th instar cabbage loopers were compared because these two larval stages have distinct feeding behaviors that result in different types of damage to the leaf. The rate of carbon assimilation, particularly at high irradiance, is closely related to the activity of Rubisco, the primary carboxylating enzyme in C_3 plants (Farquhar *et al*., 1980; von Caemmerer and Farquhar, 1981; Long and Bernacchi, 2003), and transcription of genes for Rubisco and other photosynthetic proteins can be suppressed by herbivory (Schenk *et al*., 2000; Halitschke and Baldwin, 2003; Zhu-Salzman *et al*., 2004). To determine if the expression of Rubisco contributed to the spatial pattern of photosynthesis following herbivory, a transgenic line of *A. thaliana* (L.) Heynh. was constructed that used the Rubisco small subunit promoter to drive expression of a green fluorescent protein (GFP) gene. The effect of herbivory on water loss was inferred from thermal images of leaf temperature.

Materials and methods

Transformation of A. thaliana

A fusion of the GFP gene (Chiu *et al*., 1996), modified by site-directed mutagenesis for enhanced fluorescence by making the amino acid substitutions F64L, V163A, and I167T, to the promoter for Rubisco small subunit (RbcS1A, At1g67090) was inserted into the *Agrobacterium tumefaciens* binary vector pBI101.3 (CLONTECH, Palo Alto, CA). *Arabidopsis thaliana* was transformed by floral dip (Clough and Bent, 1998) and transformants were selected on Murashige and Skoog medium (Sigma, St Louis, MO) containing kanamycin (50 mg l^{-1}) and carbenicillin (300 mg l^{-1}). Several transgenic lines were obtained. Protein was extracted from leaves from each line to estimate GFP content through a gel blot assay as in Zielinski *et al*. (1989). Because the RbcS1a::GFP transgene was inserted into different locations in the *A. thaliana* genome, each of 7 different lines expressed GFP to a different degree. Across lines, there was a strong correlation between fluorescence and the quantity of GFP in the leaf (log (GFP fluorescence intensity)=0.56×%GFP/total protein+1.33; r^2=0.82; P <0.01), where other metabolites that yield green fluorescence caused a positive y-intercept (Mantis and Tague, 2000). A transgenic line with relatively low GFP expression was chosen for herbivory experiments.

Effects of herbivory on gas exchange and fluorescence

To examine the potential coupling between fluorescence and carbon uptake, the relationship between Φ_{PSII} and apparent quantum yield of CO_2 fixation (Φ_{CO2}; net photosynthesis/incident light) was determined in the presence or absence of herbivory on ten 5-week-old plants. First and 4th instar cabbage loopers (*T. ni* (Hübner) Lepidoptera: Noctuidae) were used to generate variable levels of damage on transgenic plants. The cabbage looper is a generalist herbivore that feeds on many types of crops, including crucifers (Shorey *et al*., 1962). First instars feed on the underside of leaves; they make small holes, avoiding veins and leaving the upper epidermis intact. By contrast, 4th instars make large holes, consuming minor and major veins and the leaf epidermis. Insects were from a colony maintained by MR Berenbaum.

In an initial experiment to examine the relationship between Φ_{PSII} and Φ_{CO2}, a single leaf on each of five plants was exposed to five 1st instar larvae confined to a clip cage. Plants were grown for approximately 6 weeks at an irradiance of 190 μmol m^{-2} s^{-1} photon flux density (*PFD*) for 10 h d^{-1} at 22 °C (14 h of darkness at 20 °C). Clip cages consisting of mesh-covered plastic rings (14.5 mm internal diameter) were used to limit feeding to 165 mm^2 on the underside of one leaf per treatment plant. Leaves were large enough to cover the openings of the clip cages. Larvae remained on the leaves for ~24 h, until they had removed approximately 12% of the leaf area within the clip cage (20 mm^2). Empty clip cages were placed on leaves of the other five plants as a control. A portable photosynthesis system (LI-6400, LI-COR, Lincoln, NE, USA) with a leaf chamber fluorometer (6400-40 LCF, LI-COR) was used to measure spatially-averaged Φ_{PSII} and Φ_{CO2} at eight irradiance levels (100, 150, 200, 250, 350, 450, 550, and 650 μmol m^{-2} s^{-1}, *PFD*) under 20% oxygen (atmospheric conditions) and 0.5% oxygen (to inhibit photorespiration). The damaged leaf area with surrounding leaf tissue was placed in the measuring cuvette at 400 μl l^{-1} CO_2; gas exchange rates, measured at a flow rate of 100 μmol s^{-1}, were corrected for the amount of remaining leaf area.

Subsequent experiments with 1st and 4th instars were conducted sequentially. To examine the effect of 1st instars, ten plants were exposed to varying levels of damage (9–24% of tissue in clip cage removed). Seven 1st instars were allowed to feed on one leaf of each plant for 8–23 h. An equal number of control plants each received a clip cage. Chlorophyll and GFP fluorescence images were collected, beginning the day after herbivores were first placed on the plants (day 1). The same leaves were measured again the next day (day 2) and 2 d later (day 4). A similar set of experiments was performed with 4th instars (19–50% of tissue removed; n=10). Prior to measuring chlorophyll fluorescence, leaves were light adapted to 107 μmol m^{-2} s^{-1} *PFD* for 10 min; chlorophyll and GFP fluorescence were measured for leaves in the same orientation.

The potential mechanisms underlying the indirect reduction of photosynthesis by herbivory were further examined in another experiment where, in addition to measurements of the spatial pattern of gene expression and leaf surface temperature, chlorophyll fluorescence and gas exchange on damaged and control leaves were measured simultaneously. Five-week-old transgenic plants were exposed to 1st instar *T. ni* herbivory. Plants were grown at an incident irradiance of 110–145 μmol m^{-2} s^{-1} *PFD* for 11 h days at 22 °C (13 h nights at 20 °C). First-instar larvae were applied to one leaf on each of eight plants and removed about 11% of tissue within the clip cages (18 mm^2). Another eight control plants received empty clip cages. Measurements began the day after herbivores were first placed on the plants (day 1) and were taken again 3 d later (day 4). Near the end of the dark period after the larvae were removed, gas exchange (dark respiration and transpiration), thermal images and dark-adapted fluorescence parameters were measured. Plants were in the dark for at least 8 h prior to measurement. Variation in surface temperature across damaged and control leaves were measured with an infrared camera (ThermaCAM SC, FLIR Systems, Portland, OR). Gas exchange measurements were made with the infrared gas analyser system. The top of the leaf chamber was replaced with anti-reflection coated glass (NT46-103, Edmund Industrial Optics, Barrington, NJ) that permitted imaging of chlorophyll fluorescence. Measurements were later corrected to account for the loss of leaf area to herbivory.

The dark-adapted leaf was imaged for minimum and maximum chlorophyll fluorescence (F_o, F_m), allowed to light-adapt at 145 μmol m^{-2} s^{-1} for 45 min, and then remeasured for chlorophyll fluorescence (F', F'_m) and gas exchange. Images of GFP were collected after the measurement of chlorophyll fluorescence, using the same anti-reflection glass described above to flatten leaves.

This experiment was repeated with 4th instars and 7-week-old transgenic *A. thaliana*. Six control plants each received an empty clip cage on one leaf, while eight treatment plants received a single 4th instar larva in a clip cage on one leaf per plant.

The effects of variation in external CO_2 on gas exchange and the spatial pattern of Φ_{PSII} were examined in a subsequent experiment with 6-week-old plants and 5th instar larvae. Thirteen plants were exposed to herbivory by a 5th instar larva confined to a clip cage on 1 leaf per plant. Larvae removed varying amounts of leaf tissue (2–52% of total leaf area removed). Nine plants received empty clip cages. The round clip cages had a diameter of 2.4 cm. Chlorophyll fluorescence and gas exchange of light-adapted leaves were measured 2 d after larvae were removed at 400 μl l^{-1} CO_2 and then again after steady-state rates were achieved at 1000 μl l^{-1} CO_2.

Fluorescence imaging system

The imaging system used to measure fluorescence from chlorophyll and GFP, initially described by Zangerl *et al*. (2002), consisted of a progressive scan charge-coupled device camera (659×494, 8-bit, 10 Hz, JAI, Laguna Hills, CA, USA). The light source was a dome-shaped hood containing 1200 blue (NSPB500s, Nichia Corporation, Mountville, PA, USA) and red (HLMP-C116, Agilent Technologies, Palo Alto, CA, USA) light-emitting diode (LEDs) that provided either short, intense pulses or continuous actinic light. The light source and camera were interfaced with a computer that controlled the intensity and duration of light output, and recorded chlorophyll fluorescence emitted from the leaf. Chlorophyll was excited with blue LEDs only and the resulting fluorescence (>715 nm) was measured through a Schott glass filter (RG715, ThermoOriel, Stratford, CT, USA).

Images of chlorophyll fluorescence were used to calculate parameters related to photochemical efficiency. Images of light-adapted steady-state fluorescence (F') and light-adapted maximum fluorescence (F'_m) were captured for each leaf with custom software where pixel intensity indicated the level of fluorescence. Corresponding pixels in a pair of F' and F'_m images were used to calculate the operating photochemical efficiency of photosystem II ($\Phi_{PSII} = (F'_m - F')/F'_m$) as in Zangerl *et al*. (2002). Based on these calculations, the software constructed a spatial map of operating photochemical efficiency across the leaf in which different colours represented varying levels of efficiency. Dark-adapted chlorophyll fluorescence measurements consisted of images of minimal fluorescence (F_o) and maximal fluorescence (F_m). These images were used to calculate maximum photosynthetic efficiency ($F_v/F_m=(F_m-F_o)/F_m$). Likewise, images of non-photochemical quenching, which mostly indicated thermal dissipation of excess light energy, were calculated from images of F_m and F'_m ($NPQ=F_m/F'_m - 1$). For each pixel, Φ_{PSII}, F_v/F_m, and NPQ were expressed as values from 0 to 1.0.

To classify leaf area statistically as photosynthetically depressed, the values of the different fluorescence parameters for the treatment leaves were compared with control leaves. The mean pixel intensity and standard deviation of each control image were fitted to a normal distribution using a z-transformation to determine the lower 10% of values in the distribution. The lower 10% threshold was averaged among all of the control plants for each day of measurement to compare with damaged plants measured the same day. Leaf area was defined as damaged when pixel intensity was equal to or less than the lower 10% of the control distribution. Mean pixel intensity (*PI*) was converted to actual values of Φ_{PSII}, F_v/F_m, or NPQ using linear relationships ($y=PI/256$ for Φ_{PSII} and F_v/F_m; $y=PI/25.6$ for NPQ). The remaining leaf area was considered undamaged.

By exchanging the light source and camera filter, the imaging system was also used to measure the expression level of RbcS1a::GFP. A moveable ring of four sets of blue LEDs (NSPB 500s, Nichia Corporation, Mountville, PA, USA) each covered by

a blue light filter (XF1073, Omega Optical, Inc., Brattleboro, VT, USA) was placed around the camera, producing a focused beam of blue light (475±20 nm) that excited the GFP in the leaves. To measure GFP fluorescence, the camera was fitted with a filter that transmits green light from 535±23 nm (XF3084, Omega Optical, Inc.). GFP fluorescence images were corrected mathematically for minor variation in the light field with an image collected from a grey card using image analysis software (ERDAS IMAGINE, version 8.6, Leica Geosystems, Atlanta, GA).

Statistical analysis

The effect of herbivory and oxygen level during the gas exchange measurement on the slope of the relationship between Φ_{CO2} and Φ_{PSII} was tested by analysis of covariance, using Φ_{CO2} as the dependent variable and Φ_{PSII} as a covariate (SAS, version 8.1, Cary, NC). Slopes were considered different if a significant interaction occurred between the covariate and type of treatment (herbivory versus control or high versus low O_2). The intercept of each of the four regression lines was also analysed by linear regression (SAS, version 8.1).

Gas exchange parameters (respiration, assimilation, and transpiration in the light or dark) and chlorophyll fluorescence parameters (F_v/F_m, Φ_{PSII}, and *NPQ*) of control and treatment leaves were compared with a repeated-measure analysis of variance (SAS, version 9.1). For F_v/F_m and Φ_{PSII}, the mean values were examined for the entire leaf, the photosynthetically depressed area and the undamaged area. In addition, the percentage of photosynthetically depressed area relative to leaf area measured was also compared. Data from the experiments involving 1st and 4th instars were analysed in separate models that included the effects of herbivory and time, as well as the interaction between them.

Thermal images were used to compare temperatures in discrete regions of leaves near the edge of holes versus farther away from the holes. In images of damaged leaves, the mean temperature of three circular areas (about 0.6 mm^2) immediately adjacent to the damage and three comparably sized areas far from the damage were determined. For control leaves, leaf temperature was measured in six randomly spaced, similar-sized areas. Areas near the damage were randomly paired with areas far from the damage; the temperature difference was calculated for each of the three pairs. In control leaves, the six areas were randomly paired and the temperature difference within each pair was calculated. The difference in temperature was analysed with a repeated measures model with the plant as the unit of measurement (SAS, version 9.1).

To examine if increasing the supply of CO_2 to a damaged leaf would overcome stomatal limitation to photosynthesis, damaged and control leaves were measured at 400 $\mu l\ l^{-1}$ CO_2 and then again at 1000 $\mu l\ l^{-1}$ CO_2. A two-way ANOVA was used to compare assimilation rates at low and high CO_2 in damaged and control leaves (SAS, version 9.1). Least square differences with a Tukey-Kramer adjustment were also calculated.

Results

At 0.5% or 20% O_2, values of Φ_{PSII} were correlated with Φ_{CO2} for control leaves and those exposed to herbivory (Fig. 1). The coefficients of determination (r^2) for the four combinations of control versus herbivory and high versus low O_2 were ⩾0.98. The slopes for leaves measured at 20% O_2 were significantly lower than those at 0.5% O_2 (P <0.05). Although not statistically significant (P >0.05), the slopes of damaged leaves tended to be lower than those of controls. The y-intercepts of the regression lines were not significantly different from 0.

Holes produced by 1st instars had a substantially greater perimeter-to-area ratio than 4th instars (Fig. 2). The slopes of the linear regression lines for 1st and 4th instars were significantly different (P <0.01; 1st instars: r^2=0.76; 4th instars: r^2=0.39).

For a given area removed, 1st instars reduced photosynthetic efficiency more than 4th instars in photosynthetically depressed areas by day 4 (Fig. 3). The correlation between percentage of area removed and Φ_{PSII} was significant for both 1st and 4th instars (P <0.01; 1st instars: r^2=0.84; 4th instars: r^2=0.59), and the slope of the 1st instar correlation was seven times greater than the 4th instar slope (−0.007 versus −0.001).

Herbivory by 1st and 4th instars had different effects on the spatial pattern of chlorophyll fluorescence. The values of Φ_{PSII} and F_v/F_m for leaves damaged by 1st instars were

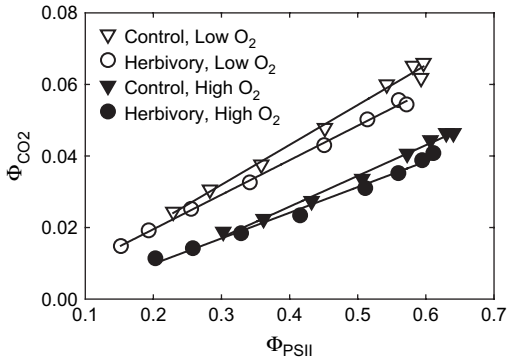

Fig. 1. Linear correlation between photosystem II operating efficiency (Φ_{PSII}) and the quantum efficiency of carbon assimilation (Φ_{CO2}) in leaves exposed to herbivory (circles) and non-damaged controls (triangles) at 0.5% and 20% oxygen (open and filled symbols, respectively). Measurements were taken at eight different light levels varying from 100 to 650 $\mu mol\ m^{-2}\ s^{-1}$. Each point represents the average value of five leaves; r^2 ⩾0.98, P >0.01.

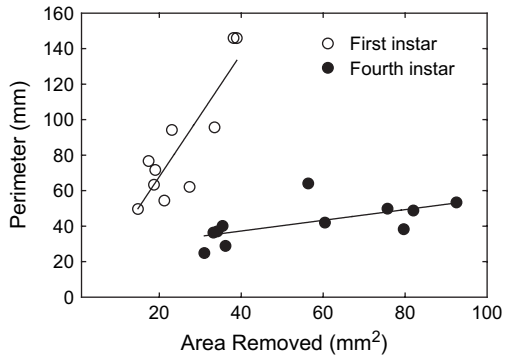

Fig. 2. The relationship between the total perimeter of cut edges to the area of leaf tissue removed by 1st instar (open circles) and 4th instar herbivory (closed circles). Each point represents a leaf on one plant, 1 d after larvae were removed.

depressed along the cut edge of holes and in the areas between some of the holes (Fig. 4). By contrast, the values of Φ_{PSII} and F_v/F_m following 4th instar herbivory were uniformly high across the leaf with depressed values evident only in a thin band immediately adjacent to the hole (Fig. 4). When the hole was close to the outer edge of the leaf, a small portion of the tissue between the hole and edge also exhibited depressed F_v/F_m.

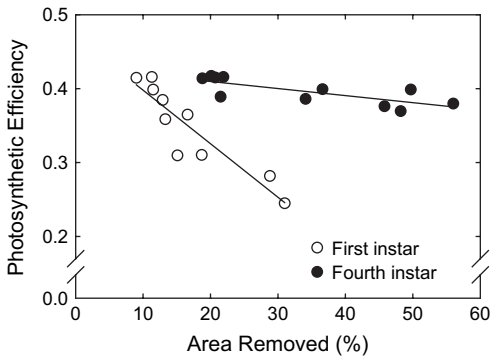

Fig. 3. Correlation between the proportion of leaf tissue removed (relative to area measured) and photosystem II operating efficiency (Φ_{PSII}) of the areas of the leaf characterized as photosynthetically depressed. Leaf area with Φ_{PSII} within or below the lower 10% of the average distribution for control leaves was considered photosynthetically depressed. Each point represents a leaf on one plant, 4 d after it was exposed to herbivory.

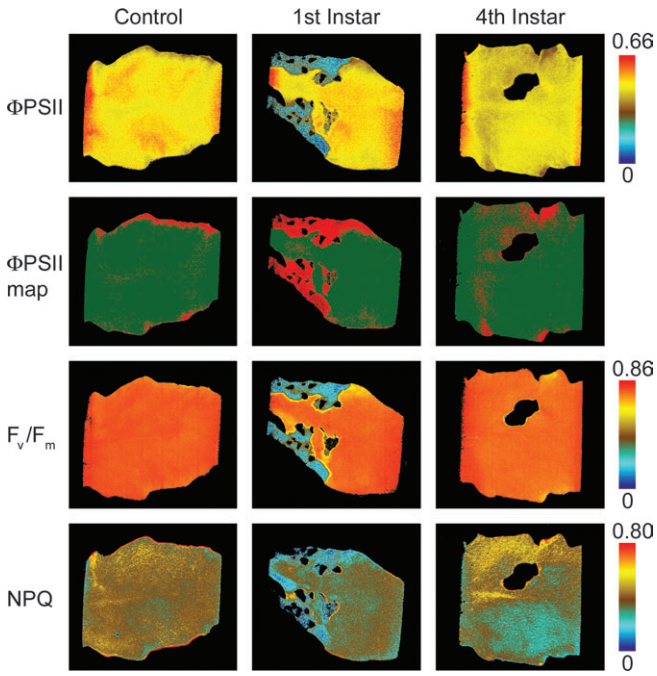

Fig. 4. False colour images representing the spatial variation in the photosystem II operating efficiency (Φ_{PSII}), maximum photosynthetic efficiency (F_v/F_m), and non-photochemical quenching (*NPQ*) of fluorescence for a control leaf (left) and a leaf exposed to 1st instar herbivory (middle) or 4th instar herbivory (right). These images were taken 4 d after herbivory had occurred. The Φ_{PSII} map shows the photosynthetically depressed area in red and the undamaged area in green. Leaf tissue operating at or below the lower 10% of the control Φ_{PSII} distribution was categorized as photosynthetically depressed, while the remaining area was considered undamaged. The black areas within the damaged leaf represent holes.

Herbivory by 1st and 4th instars had different effects on net carbon assimilation (Table 1). One day after herbivory, leaves eaten by 1st instars had lower net carbon assimilation than controls; this difference disappeared by day 4. Herbivory by 4th instars had no effect on gas exchange on either day. Herbivory by both 1st and 4th instars caused greater respiration in damaged leaves compared to controls on day 1, and this stimulation persisted for 4 d in leaves damaged by 4th instars. Both instars caused greater transpiration in the dark on day 1, but the difference disappeared by day 4 (Table 1; treatment×day interaction for 4th instars: P=0.02, F=6.13).

Water lost from the cut edge of holes made by the herbivores probably accounted for the increase in transpiration in the dark over the 4 d measuring period. There was no significant effect of herbivory on transpiration in the light for 1st instars (Table 1), but leaves damaged by 4th instar caterpillars had slightly greater transpiration in the light than control leaves on day one (Treatment effect for 4th instar caterpillars on day one: P=0.08, F=3.43).

First instar larvae caused a substantial decrease in the dark-adapted F_v/F_m in areas of the leaves classified as having depressed photosynthesis on the first day following their removal, and the area of depressed F_v/F_m was approximately three times greater than in control leaves (Table 2). The statistically significant treatment by day effect (P=0.04, F=4.91) indicated that for herbivory by 1st instar larvae the depression of F_v/F_m deepened with time. The reduction of F_v/F_m by 1st instar larvae in damaged portions of the leaf was sufficiently large that it reduced the spatially averaged value for the entire leaf (Table 2).

In areas of the leaf where herbivory by 1st instar larvae decreased Φ_{PSII}, values of *NPQ* typically increased (data not shown). However, this was not always the case. In areas of the leaf that became visibly desiccated following

Table 1. *Least square means (±SE) are presented for the rate of dark respiration (R_d), assimilation (A), and transpiration in the light (Tr–light) and in darkness (Tr-dark) in control leaves and leaves exposed to 1st or 4th instar herbivory, 1 d and 4 d after herbivory*

A significant difference (P <0.05) between a control and eaten leaf for a particular day is indicated with an asterisk.

			R_d	A	Tr-light	Tr-dark
1st instar	Day 1	C	−0.56±0.07*	6.4±0.3*	1.7±0.1	0.36±0.10*
		E	−0.76±0.07	5.4±0.3	1.9±0.1	0.78±0.09
	Day 4	C	−0.49±0.07	5.9±0.3	1.7±0.1	0.42±0.09
		E	−0.63±0.07	5.3±0.3	1.5±0.1	0.61±0.09
4th instar	Day 1	C	−0.60±0.04*	6.8±0.2	1.7±0.1*	0.45±0.06*
		E	−0.76±0.03	6.6±0.2	2.0±0.1	0.83±0.06
	Day 4	C	−0.42±0.04*	6.5±0.2	1.6±0.1	0.38±0.06
		E	−0.56±0.03	6.4±0.2	1.6±0.1	0.46±0.06

Table 2. *Least square means (±SE) are presented for the mean* F_v/F_m *and* Φ_{PSII} *of the whole leaf, photosynthetically depressed area and undamaged area, as well as the percentage of depressed area relative to area measured*

A significant difference (P <0.05) between a control and eaten leaf for a particular day is indicated with an asterisk.

				Whole	Depressed	Undamaged	Depressed area
F_v/F_m	Ist instar	Day 1	C	0.77±0.004*	0.73±0.02*	0.77±0.002	11.8±3.0*
			E	0.74±0.004	0.68±0.02	0.77±0.002	31.5±3.0
	Ist instar	Day 4	C	0.78±0.01*	0.73±0.02*	0.78±0.002	7.1±3.0*
			E	0.72±0.01	0.59±0.02	0.78±0.002	25.7±3.0
	4th instar	Day 1	C	0.78±0.005	0.74±0.003	0.78±0.003	11.7±6.9
			E	0.77±0.004	0.73±0.003	0.78±0.003	16.1±6.0
	4th instar	Day 4	C	0.78±0.005	0.74±0.003*	0.78±0.003	13.1±6.9
			E	0.77±0.004	0.73±0.003	0.78±0.003	27.4±6.0
Φ_{PSII}	Ist instar	Day 1	C	0.47±0.006	0.39±0.02*	0.48±0.004	10.5±3.0
			E	0.46±0.006	0.35±0.02	0.49±0.004	18.7±3.0
	Ist instar	Day 4	C	0.48±0.006	0.38±0.02	0.49±0.004	8.6±3.0
			E	0.46±0.006	0.34±0.02	0.49±0.004	17.1±3.0
	4th instar	Day 1	C	0.45±0.005	0.39±0.002	0.46±0.004	11.5±3.0
			E	0.44±0.004	0.38±0.002	0.45±0.003	16.4±2.6
	4th instar	Day 4	C	0.45±0.005	0.38±0.002*	0.45±0.004	10.9±3.0*
			E	0.43±0.004	0.37±0.002	0.45±0.003	19.5±2.6

herbivory, *NPQ* was low. When *NPQ* was averaged over the leaf, areas damaged by 1st instars had higher *NPQ* than controls on day 1 (0.46±0.01 versus 0.40±0.01), but this difference disappeared by day 4. There was a significant treatment×day interaction (P=0.02, F=6.43).

Herbivory by 4th instars also reduced Φ_{PSII} and F_v/F_m in regions of the leaf classified as photosynthetically depressed (Table 2). Although the response to 1st and 4th instar larvae could not be compared statistically because they were independent experiments, the magnitude of the reduction and the area affected appeared to be less when leaves were damaged by 4th instar larvae than by 1st instars. *NPQ* was significantly greater in leaves damaged by 4th instars than controls on day 1 (0.53±0.01 versus 0.49±0.02), and this difference disappeared by day 4.

As a result of evaporative cooling, herbivory caused significant decreases in leaf temperature near the cut edges (Fig. 5). For leaves damaged by 1st instar larvae, area near the cut edge was 0.60±0.15 °C cooler than areas away from the damage on day one (P <0.01, F=19.3, n=4) and 0.28±0.10 °C cooler on day 4 (P=0.01, F=6.7, n=8). For leaves damaged by 4th instars, the area near the cut edge was 0.35±0.08 °C cooler than that away from the damage on day 1 (P <0.01, F=11.2, n=8), and differences in temperature across different regions of the leaf could not be resolved statistically by day 4 (P=0.94, F=0.01, n=8). A significant day×treatment effect for herbivory by 1st instars (P=0.04, F=4.4, n=24) and 4th instars (P=0.02, F=5.8, n=28) revealed that the difference in temperature between regions near and away from the cut edges diminished with time. In control leaves, there were no statistically significant differences in leaf temperature between random locations on any day.

In some cases, herbivory by large caterpillars (5th instar) caused a reduction in Φ_{PSII} in nearby leaf tissue (Fig. 6). In the cases were this occurred, the localized reductions in

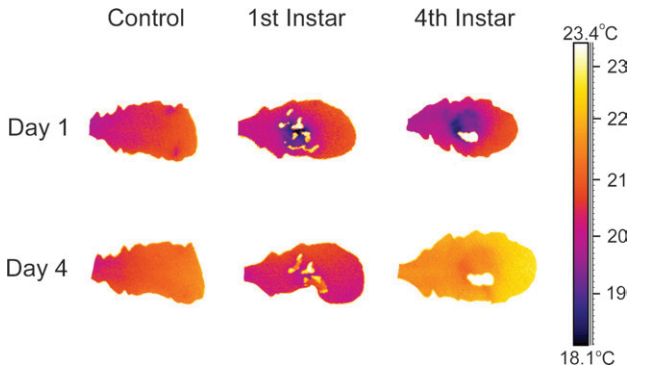

Fig. 5. False colour images of the spatial variation in temperature for a representative control leaf (left) and leaves exposed to 1st or 4th instar herbivory (middle and right, respectively) 1 d and 4 d after herbivory had occurred (top and bottom, respectively).

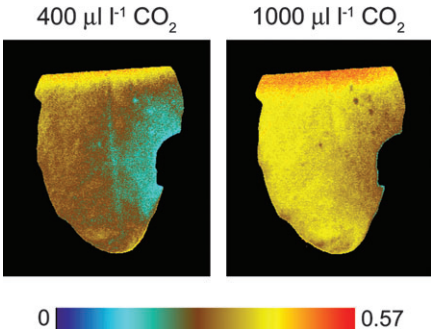

Fig. 6. Representative false colour images of the operating photosystem II efficiency (Φ_{PSII}) for a 6-week-old *A. thaliana* leaf damaged by a 5th instar *T. ni*. The images were taken 2 d after the caterpillars were removed from the leaves. The leaf was measured at 20% oxygen with 400 µl l^{-1} CO_2 (left) and then with 1000 µl l^{-1} CO_2 (right). The actinic light was 225 µmol m^{-2} s^{-1}, and the colour bar at the bottom represents the range of values for Φ_{PSII}.

Φ_{PSII} were partially reversed by exposure to elevated atmospheric CO_2 (Fig. 6). The rate of CO_2 uptake was lower for damaged than for control leaves (7.10 ± 0.41 versus 8.89 ± 0.49 μmol CO_2 m^{-2} s^{-1}, P=0.04) and both were increased by raising the external CO_2 concentration from 400 μl l^{-1} to 1000 μl l^{-1}. However, the treatment× CO_2 interaction gas exchange was not significant, indicating that alleviation of low stomatal conductance and presumably low intercellular CO_2 concentration was only partially responsible for localized depressions of Φ_{PSII}.

Neither 1st nor 4th instar herbivory appeared to change the pattern of GFP fluorescence (data not shown), indicating that herbivory did not affect the expression of the gene coding for the small subunit of Rubisco (RbcS1a). A reduction in GFP fluorescence was detected only in areas of the leaf that had become severely desiccated.

Discussion

Chewing damage by cabbage loopers reduced photosynthesis away from the leaf tissue that had been consumed, and the magnitude of this indirect suppression depended on insect feeding behaviour. First instars caused a deeper depression in the rate of carbon assimilation and Φ_{PSII} than 4th instars, potentially by accelerating the rate of water loss near damaged tissue and inducing water stress and subsequent stomatal closure. Although spatially resolved estimates of leaf water potential were not measured, 1st instars produced damage with a higher perimeter-to-area-removed ratio, resulting in more cut edge through which water was lost. The amount of cut edge is important, because tissue near the edge may have lower photosynthesis, resources may be diverted to repair the edge, and disruption of the vascular system along the edge may prevent the flow of water and photosynthate (Morrison and Reekie, 1995).

The large-perimeter-small-area holes produced by 1st instars caused a ~16% reduction in spatially integrated rates of carbon assimilation across the leaves, while 4th instars had no effect. In apple leaves, for an equivalent amount of area removed, small holes also reduce photosynthesis more than large holes (Hall and Ferree, 1976). The type of damage also affects how soybean (*Glycine max*) leaves respond to tissue removal. There is little or no change in photosynthesis with mechanical defoliation (Peterson and Higley, 1996; Aldea *et al*., 2005), while photosynthesis decreases after exposure to the Mexican bean beetle (*Epilachna varivestis*), which selectively rasps then consumes interveinal tissue (Peterson *et al*., 1998).

At a given absorbed irradiance, Φ_{PSII} represents the electron flux through the photosystem II reaction centre (Genty *et al*., 1989; Baker and Oxborough, 2005) and, given the correlation between Φ_{PSII} and Φ_{CO2} (Fig. 1), spatially discreet reductions in Φ_{PSII} (Fig. 4) contributed to reduced rates of net carbon assimilation following herbivory by 1st instars. The substantial decrease in Φ_{PSII} at a given Φ_{CO2} under conditions of low oxygen represents a reduction in the allocation of electrons to photorespiration. While there was a trend of lower Φ_{CO2}/Φ_{PSII} ratio following herbivory, this difference could not be resolved statistically. In the absence of a significant effect on the relationship between Φ_{PSII} and Φ_{CO2} (Fig. 1), herbivory directly reduced Φ_{PSII}, possibly by restricting the water supply to damaged areas.

Feeding by 1st and 4th instars substantially increased transpiration in the dark on the first day following herbivory (Table 1). Because stomata were closed (stomatal conductance (H_2O)=0.033 ± 0.011 (SD) mol m^{-2} s^{-1}, n=29), a large portion of transpiration at this time was from water lost directly from the cut edges of the leaf. That the area of the leaf proximal to physical damage was cooler than distal areas further indicated that enhanced rates of transpiration were associated with the cut edges (Fig. 5). Ostlie and Pedigo (1984) also found that transpiration from soybean leaves subject to herbivory was linearly related to the length of the cut edges. Lateral movement of liquid water through the apoplast or as vapour through intercellular spaces in the mesophyll contributes to non-stomatal water loss from damaged leaves (Barbour and Farquhar, 2003; Aldea *et al*., 2005) and possibly the development of localized regions of low water potential following herbivory. Deposition of suberin at wound sites may have contributed to the cessation of this uncontrolled water loss by the fourth day following herbivory (Kolattukudy, 1980; Hiraga *et al*., 2001).

The ability partially to reverse the depression of Φ_{PSII} following herbivory by increasing external CO_2 concentration and the elevation of non-photochemical quenching (*NPQ*) in areas with low Φ_{PSII} were consistent with localized reductions in stomatal conductance. Because of the small size of damaged leaves and profligate water loss from the cut edges, it was not possible to estimate stomatal conductance near the wounds. However, in plants damaged by 5th instars, increasing the concentration of CO_2 in the vicinity of the leaf partially reversed the down-regulation of Φ_{PSII} near the damage (Fig. 6). This may indicate that low stomatal conductance and a corresponding decrease in intercellular CO_2 contributed to the decrease in Φ_{PSII}. However, the contribution of CO_2 diffusing in from the cut edges was not estimated, and it is not uncommon, particularly in homobaric leaves, for lateral diffusion of CO_2 to influence the spatial pattern of gas exchange (Jahnke and Krewitt, 2002; Pieruschka *et al*., 2005).

That regions of the leaves near damage typically had high values of *NPQ* is further evidence that localized reductions in stomatal conductance contributed to the inhibition of Φ_{PSII}. *NPQ* reflects competition, at the level of exciton transfer in the pigment bed, between thermal dissipation and photochemistry, and removes excess excitation energy, preventing damage to the photosynthetic apparatus (Krause

and Jahns, 2004). *NPQ* is a response to development of an excess pH gradient across the chloroplast membrane, and therefore requires an active photosynthetic apparatus. The build-up of the pH gradient reflects backpressure from physiology, typically the loss of sinks for ATP and NADPH due to lowered intracellular CO_2 levels, and an increase in *NPQ* often accompanies the reduction in stomatal conductance associated with drought (Omasa and Takayama, 2003; Souza *et al*., 2004).

Regions of leaves with reduced Φ_{PSII} following herbivory by 1st instars also exhibited lower values of dark-adapted F_v/F_m. Since the plants were dark-adapted for 9–16 h, there should have been sufficient time for the repair of photodamage that may have occurred to the photosynthetic apparatus (Krause and Jahns, 2004) and dark-adapted non-radiative decay should have decreased to a minimum. That the observed reduction in F_v/F_m was associated with an increase in F_o and hence a loss of F_v, suggest that herbivory caused a decrease in the rate constant of photochemistry rather than physical damage leading to dissociation of light harvesting complex II (or other chlorophyll complexes) from the photosynthetic apparatus (Oxborough, 2004).

Herbivory caused a 27–36% increase in dark respiration that lasted for several days. Enhanced respiration may be a wound response or relate to accelerated production of inducible defensive chemicals (Zangerl *et al*., 1997). The rate of mitochondrial respiration typically is 1.2–4.3-fold greater in the dark than in the light (Atkin *et al*., 2000), but even these greater night-time values were not sufficient to explain fully the reduction in carbon assimilation caused by 1st instars (Table 1).

In contrast to 1st instars, feeding by 4th instars had little impact on chlorophyll fluorescence. Although their herbivory significantly affected F_v/F_m, Φ_{PSII}, and *NPQ*, the magnitudes of these effects were small. Leaves damaged by 4th instars were still green and healthy by day 4, although small areas of the leaf located between the leaf margin and a nearby hole often became withered.

Cutting veins disrupts transport of water and photosynthate, and is likely to cause water stress and inhibit photosynthesis. Nevertheless, the damage by 4th instars, which involved severing many veins, was less detrimental to photosynthesis than the dispersed pattern of 1st instar feeding which avoided veins. The reticulate vasculature of *A. thaliana* allows water to move around sites of damage to supply nearby tissue (Sack *et al*., 2004). This hydraulic redundancy may more effectively compensate for one large hole than many small holes that result in islands of tissue isolated from the rest of the leaf.

Neither 1st nor 4th instars affected RbcS1a::GFP fluorescence, suggesting that expression of the Rubisco small subunit gene was not measurably affected by larval damage. Schenk *et al*. (2000) found that expression of Rubisco decreases 5-fold in *A. thaliana* after exposure to methyl jasmonate, a signalling molecule that influences defence-related genes and is critical for insect defence in *A. thaliana* (McConn *et al*., 1997). The amount of Rubisco small subunit transcripts decreases when *A. thaliana*, tomato (*Lycopersicon esculentum*) and rice (*Oryza sativa*) are exposed to drought (Bartholomew *et al*., 1991; Williams *et al*., 1994; Vu *et al*., 1999). It seems likely that wilting caused by herbivory-induced water loss should also reduce the number of Rubisco small subunit transcripts. However, the stability of GFP (Sheen *et al*., 1995) prevented a decrease in expression of the Rubisco small subunit gene from being detected.

The proliferation of specialized feeding habits among insect taxa (Labandeira *et al*., 1994) may produce highly variable spatial patterns of photosynthesis following herbivory (Welter, 1989). While the taxonomic diversity and number of feeding habits among insects is high, the mechanisms of injury are not unique to individual species and can be grouped into guilds (Peterson, 2000). Among foliverous insects, potential injury guilds include: mandibulate feeders (Orthoptera, and most Coleoptera, Hymenoptera, and Lepidoptera) which feed externally and indiscriminately sever veins; leaf miners (some Coleoptera, Lepidoptera, Hymenoptera, and Diptera) which feed only on parenchyma cells between epidermal layers; pit feeders (Thysanoptera) which rasp small discontinuous holes into leaf surfaces; and skeletonizers (Lepidoptera, Coleoptera) which consume only interveinal tissue, leaving vascular tissues intact. In addition, different larval stages within a species may inflict different types of damage.

In this study, a dispersed pattern of damage that produced holes with high perimeter-to-area ratio was more detrimental to photosynthesis than large holes with a low perimeter-to-area ratio, and the difference seemed to be related to the amount of water stress produced. The impact of herbivory on photosynthesis is controlled not only by the amount of tissue removed, but where and how the tissue is removed (Higley *et al*., 1993). A better understanding of how the mode of tissue removal affects photosynthesis may also help identify agricultural pests that significantly decrease photosynthesis and potentially lower crop yields.

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