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Original Article

Herbivore perception decreases photosynthetic carbon assimilation and reduces stomatal conductance by engaging 12-oxo-phytodienoic acid, mitogen-activated protein kinase 4 and cytokinin perception

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ABSTRACT

Herbivory-induced changes in photosynthesis have been documented in many plant species; however, the complexity of photosynthetic regulation and analysis has thwarted progress in understanding the mechanism involved, particularly those elicited by herbivore-specific elicitors. Here, we analysed the early photosynthetic gas exchange responses in *Nicotiana attenuata* plants after wounding and elicitation with *Manduca sexta* oral secretions and the pathways regulating these responses. Elicitation with *M. sexta* oral secretions rapidly decreased photosynthetic carbon assimilation (A_C) in treated and systemic (untreated, vascularly connected) leaves, which were associated with changes in stomatal conductance, rather than with changes in Rubisco activity and 1-5 ribulose-1,5-bisphosphate turnover. Phytohormone profiling and gas exchange analysis of oral secretion-elicited transgenic plants altered in phytohormone regulation, biosynthesis and perception, combined with micrografting techniques, revealed that the local photosynthetic responses were mediated by 12-oxo-phytodienoic acid, while the systemic responses involved interactions among jasmonates, cytokinins and abscisic acid signalling mediated by mitogen-activated protein kinase 4. The analysis also revealed a role for cytokinins interacting with mitogen-activated protein kinase 4 in CO_2-mediated stomatal regulation. Hence, oral secretions, while eliciting jasmonic acid-mediated defence responses, also elicit 12-oxo-phytodienoic acid-mediated changes in stomatal conductance and A_C, an observation illustrating the complexity and economy of the signalling that regulates defence and carbon assimilation pathways in response to herbivore attack.

Key-words: abscisic acid; CO_2 regulation; gas exchange; herbivore-associated elicitors and herbivore-especific responses; herbivore perception; jasmonates; photosynthesis.

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INTRODUCTION

Biotic stress, such as herbivory, compromises plant growth and fitness (Rosenthal and Kotanen, 1994; van Dam and Baldwin, 1998; Huot *et al.*, 2014). Growth and fitness depend largely on a plant's capacity to assimilate carbon from photosynthetic reactions. Responses in photosynthesis elicited by herbivore attack have been investigated in a number of plants species (Welter, 1989; Nabity *et al.*, 2009), commonly studied in the context of the different insect-feeding guilds (e.g. chewing and piercing damage) as a way to facilitate predictions on the basis of the physiological damage caused by the attack (Peterson, 2000). However, predictions about photosynthetic responses to herbivore attack have been met with mixed success, even within the responses elicited by an attack from herbivores of the same guild and even the same species (Peterson and Higley, 1996; Aldea *et al.*, 2005, Peterson *et al.*, 1998, Tang *et al.*, 2006). Thus, photosynthetic responses appear to be highly herbivore/stage specific (Baldwin, 1990).

The study of inducible resistance has greatly enriched our understanding of herbivore-specific responses. It is now well known that specific herbivore-associated elicitors (henceforth, 'herbivore elicitors'), commonly restricted to a particular plant–insect interaction, allow plants to differentiate herbivore attack from wounding their feeding causes, leading to the activation of herbivore-specific responses (Halitschke *et al.*, 2001; Bonaventure, 2012; Acevedo *et al.*, 2015). Photosynthetic responses elicited by herbivore elicitors have been observed for piercing-sucking herbivores. For instance, photosynthetic rates increased after the injection of saliva extracts of *Tupiocoris notatus* (Miridae) into the spongy parenchyma cells of leaves of *Nicotiana attenuata* plants (Halitschke *et al.*, 2011). Interestingly, this was not observed when *Manduca sexta* saliva extracts were injected. However, the degree of carboxylation efficiency in the leaves of *Datura wrightii* plants when plants were attacked by wild-caught and laboratory-reared populations of *M. sexta* (Sphingidae) larvae were observed to differ (Barron-Gafford *et al.*, 2012). These differences may reflect differences in the composition of herbivore elicitors in the oral

secretions of wild-caught and laboratory-reared larvae and perhaps require additional inputs from the damage of the plant's cellular integrity resulting from wounding by chewing herbivores.

The mechanisms leading to changes in photosynthesis after herbivore attack are multifaceted and include both direct and indirect effects (Nabity *et al.*, 2009). Photosynthesis can clearly be directly affected by the removal of photosynthetic tissue and damage to the vascular tissues that affects the integrity of water and nutrient flux required to support photosynthesis (Aldea *et al.*, 2005; Sack *et al.*, 2013). However, herbivory may also influence photosynthesis indirectly by changing the accumulation of sugars (Gifford and Evans, 1981; Peterson, 2000; Thompson *et al.*, 2003; Schultz *et al.*, 2013), increasing the accumulation of secondary metabolites (Baldwin and Callahan, 1993; Gog *et al.*, 2005) and changing photosynthesis-related genes (Giri *et al.*, 2006; Bilgin *et al.*, 2010); these changes are all part of the large-scale reconfiguration of metabolism that occurs when herbivores attack plants (Schwachtje and Baldwin, 2008; Nabity *et al.*, 2013). Herbivore elicitors are known to elicit changes in the transcripts of photosynthetic genes, sugars and secondary metabolites (Giri *et al.*, 2006, Bilgin *et al.*, 2010; Philippe *et al.*, 2010; Ferrieri *et al.*, 2015) and the levels of several stress-related plant hormones (Vadassery *et al.*, 2012; Dinh *et al.*, 2013; Vos *et al.*, 2013; Schafer *et al.*, 2015a); however, which of these many changes contribute to the changes in photosynthetic physiology are not well understood.

The best-studied hormone regulating induced resistance to herbivore attack is jasmonic acid (JA) and its metabolites (Geyter *et al.*, 2012). JA biosynthesis begins in the chloroplasts with the oxidation of polyunsaturated fatty acids (e.g. α-linolenic acid) by lipoxygenases (13-LOX) and their subsequent cyclization and oxidation by allene oxide cyclase (AOC) and synthase to yield 12-oxo-phytodienoic acid (OPDA). OPDA is transported to the peroxisome to undergo a series of redox reactions involving OPDA-reductase and acyl-CoA transferase (ACX1) to produce JA (Wasternack and Hause, 2013). JA is then conjugated to isoleucine to yield JA-isoleucine (JA-Ile) in the cytoplasm, which is thought to be the main active compound in JA-mediated defence signalling (Gfeller *et al.*, 2010), but a signalling function for the important biosynthetic intermediate, OPDA, has also been reported (Bosch *et al.*, 2014).

Studies using transgenic lines impaired in the biosynthesis and perception of JA signalling suggest that JAs are an important regulator of photosynthetic responses to herbivory. While changes in dark-adapted electron transport are induced 24 h after *M. sexta* herbivory to wild type (WT) *N.* attenuata plants, they are lacking in JA-deficient as *LOX* transgenic plants (Nabity *et al.*, 2013). Elicitation with coronatine (a JA-Ile analogue) delayed stomatal closure after sunset, thus prolonging carbon assimilation (Attaran *et al.*, 2014). However, recent publications have also highlighted the importance of phytohormones other than JAs in mediating responses to herbivore attack, including abscisic acid (ABA) and cytokinins (CKs), also involved in biochemical and physiological aspects of photosynthetic regulation (Erb *et al.*, 2012; Schafer *et al.*, 2015b; Chaves *et al.*, 2003; Farooq *et al.*, 2009; Pinheiro and Chaves, 2011; Wilkinson *et al.*, 2012; Acharya and Assmann, 2009; Hu *et al.*, 2013; Misra *et al.*, 2015).

One of the best described model systems for herbivory-induced responses and signalling is *N. attenuata* and its interaction with *M. sexta* larvae. *N. attenuata* specifically responds to the herbivore elicitors contained in the oral secretions of *M. sexta* larvae, such as FACs (Halitschke *et al.*, 2001). Application of *M. sexta* oral secretions to leaf wounds ('OS-elicitation') accurately mimics herbivore responses in local and systemic tissues, which includes the accumulation of defence metabolites, changes sugar levels and transcripts of photosynthesis-related genes (Halitschke *et al.*, 2001; Giri *et al.*, 2006; Bonaventure, 2012; Erb *et al.*, 2012; Fukumoto *et al.*, 2013; Dinh *et al.*, 2013; Benikhlef *et al.*, 2013; Chuang *et al.*, 2014 Ferrieri *et al.*, 2015; Acevedo *et al.*, 2015). Changes in sugar levels may affect photosynthesis by modifying the supply of substrates for carbon assimilation, such as ribulose-1,5-bisphosphate (RuBP) turnover or triose-phosphate (TPU) use (Fig. 1; pathway 1). Elicitation with oral secretions-related herbivore elicitors is also known to decrease the transcript accumulation of RuBP-carboxylase/oxygenase (Rubisco) and Rubisco activase (Gilardoni *et al.*, 2010; Bilgin *et al.*, 2010) and may directly affect carbon assimilation by reducing the rate of carboxylation (Fig. 1; pathway 2). OS-elicitation through its effects on several hormonal pathways, such as JA, ABA and CK (Stitz *et al.*, 2011; Dinh *et al.*, 2013; Schafer *et al.*, 2015b), may also induce changes in photosynthesis-related physiological processes, such as stomatal conductance (g_s)-reducing intracellular CO_2 concentrations (C_i) and limiting Rubisco carbon assimilation (Fig. 1; pathway 3; Farquhar and Sharkey, 1982). OS-elicitation may also induce long-lasting changes in the photosynthesis of unattacked, systemic leaves of attacked plants, which undergo a reversal of senescence as a means of compensating for the loss of photosynthesis in attacked leaves (Baldwin and Ohnmeiss, 1994; Stowe *et al.*, 2000).

Here, we examine the inference that OS-elicitation induces changes in photosynthetic gas exchange, modulated by phytohormones in *N. attenuata* plants. Because OS-elicitation rapidly changes phytohormone concentrations, we focus on the short-term changes in photosynthesis in local and systemic leaves, which are more likely to be regulated by phytohormone signalling events, than the long-term changes that likely recruit developmental responses. We use previously characterized isogenic transgenic lines of *N. attenuata* impaired in JA biosynthesis, mitogen-activated protein kinase 4 (MAPK4), ABA and CK signalling to identify the relevant signalling pathways. The analysis revealed that OS-elicitation reduces CO_2 assimilation together with stomatal conductance and identified OPDA as the main regulator of these responses in local leaves. The photosynthetic responses in systemic leaves were found mediated by a complex interaction among ABA-MPK4, CK and JA signalling. As such, the analysis provides a framework for understanding the regulation of photosynthetic gas exchange responses elicited by herbivore perception.

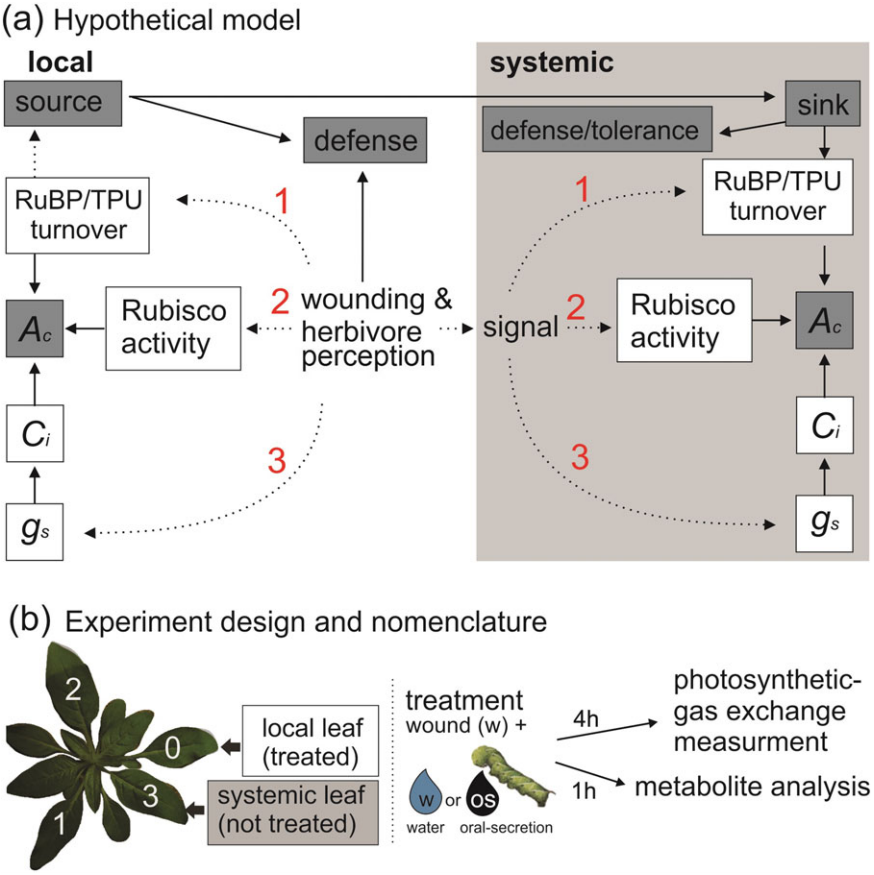

Figure 1. Three hypotheses for changes in photosynthetic capacity after oral secretion (OS) elicitation and the experimental design and nomenclature were used in this study. (a) Model of three hypothetical alternatives paths (numbered, dashed lines) by which photosynthetic parameters analysed by gas exchange could be changed in leaves elicited (local) by herbivore elicitors applied to fresh wounds and in undamaged (systemic) leaves of elicited plants. Wounding and herbivore perception can affect carbon assimilation (A_C) by either changing (1) ribulose bisphosphate regeneration and triose-phosphate utilization (RuBP/TPU turnover); (2) Rubisco activity; or (3) stomatal conductance (g_s) influencing the pool of intracellular CO_2 (C_i). Changes in photosynthetic capacity are thought to be important for defence/tolerance responses and to occur not only in locally attacked leaves but also in systemic unattacked tissues. Solid arrows and grey boxes represent relations and changes described in the literature. (b) Experimental design. Leaves of *Nicotiana attenuata* plants were wounded with a pattern wheel (W) and water (-W) or *Manduca sexta*, OS were immediately applied to the puncture wounds and photosynthetic responses were measured in treated (local, 0) leaves and orthostichous (connected by vasculature) non-treated (systemic, 3) leaves. Leaves of untreated plants of the same age were used as controls for all experiments. For metabolite analysis, leaf samples were collected 1 h after treatment from a different set of plants treated in parallel. The white numbers on leaves of *N. attenuata* plant indicate the phyllotactic relationships among the leaves (local, 0; systemic, 3) analysed. [Colour figure can be viewed at wileyonlinelibrary.com]

MATERIALS AND METHODS

Plant growth and micrografting

Experiments were conducted with the 31st inbred generation of *N. attenuata* Torr. ex S. Watson plants (Solanaceae; seeds collected from DI Ranch, UT, USA; Baldwin *et al*, 1998) (WT) and the following *N. attenuata* stably transformed lines of the same inbred generation that have been previously characterized: irAOC, irACX1, irOPR3 (line number A-07-457, A-11-170, A-07-498; Kallenbach *et al*, 2012), irMPK4 (line number A-08-119; Hetterhausen *et al*. 2012) and irCHK2/3 (A-12-356; Schäfer *et al*. 2015). Crossing irMPK4 with irCHK2/3 was performed by removing anthers from irCHK2/3 before pollen maturation and pollinating the stigma with irMPK4 pollen.

Seed germination and growth under glasshouse conditions were performed as described by Krugel *et al*., (2002). Briefly, seeds were germinated on cell culture plates with 20 mL of Gamborg's B5 (Sigma) medium with 0.5% w/v plant agar (Duchefa) and maintained in growth chambers (Percival, Perry, IA, USA) under 16/8 h light/dark regimen at 26 ± 2 °C. Ten to twelve days after germination (DAG), the seedlings were transferred to TEKU JP-3050-104 pots and later (20–22 DAG) to 1 L pots with substrate (Fruhsdorfer Nullerde, 0.5 g L^{-1} PG mix, 0.9 g L^{-1} superphosphate, 0.35 g L^{-1} $MgSO_4$*7 H_2O, 0.055 g L^{-1} Micro Max). After potting, plants were kept under glasshouse conditions with 26–28 °C under 16/8 h light (Master Sun-T PIA Agro 400, Philips, Turnhout, Belgium)/dark regime and fertilized by flood irrigation with 0.6 g L^{-1} $Ca(NO_3)_2 \cdot 4H_2O$ (Merck) and 0.3 g L^{-1} Ferty B1 (Planta Düngemittel). Experiments were conducted with plants at the rosette stage of growth (30–32 DAG).

Micrografting of WT shoots on irMPK4 roots was performed using a stereomicroscope under sterile conditions as described by Fragoso *et al*. (2011) with 8–10 DAG seedlings of similar

stem thickness. Micrografting efficiency was around 80%, and differences in growth of homografted (WT/WT and irMPK4/irMPK4) controls of the same genotype were not found, as previously described (Fragoso *et al.*, 2011). WT/irMPK4 graft combinations were chosen to dissect MPK4's role on ABA signalling as distinct from its role in the shoot.

Collection of *Manduca sexta* oral secretions

Oral secretions were collected from larvae in the third to fifth instars of two populations of *M. sexta L.* (Sphingidae): (1) laboratory-reared larvae, reared for more than 200 generations on artificial diet (as described in Koenig *et al.* 2015) and (2) larvae from eggs collected from natural ovipositions on *D. wrightii* plants at our field station (37°16 10.49″ N/113°16 53.15″ W, UT, USA, 2014). For oral secretion collection, both colony and wild larvae were hatched and fed on *N. attenuata* plants. Oral secretion was collected with a vacuum cold trap and frozen immediately until used. The oral secretions were thawed and diluted 1:5 in distilled water just before their application to fresh leaf puncture wounds. Experiments were conducted with oral secretions of laboratory-reared larvae unless otherwise specified.

Experimental design and treatments

Standardized wound treatments of *N. attenuata* leaves were performed by rolling a fabric pattern wheel three times on each side of a leaf and immediately applying 20 μL of water to the punctured holes. Herbivory was simulated by applying 1:5 diluted *M. sexta* oral secretions. In all experiments, three fully developed rosette leaves were treated. Treatments were performed 4–5 h after dawn (0500 h), when circadian regulation of photosynthesis and A_C in *N. attenuata* plants grown in the glasshouse was known to be at full capacity.

Fully expanded leaves at positions 6 to 8 (leaves −1, 0, 1 from the phyllotaxic relationships; Fig. 1b) of rosette plants were treated, and gas exchange measurements were performed 4–6 h after treatments in a treated (local) and one vascularly connected undamaged (systemic) leaf of treated and control plants ($n = 6$). Leaf lamina tissue (local and systemic) was harvested 1 h after treatment from a different experimental set of plants treated in parallel to the plants used for photosynthetic characterizations of phytohormone responses ($n = 5$). The harvested tissue was immediately frozen in liquid nitrogen and stored at −80 °C until metabolite analysis.

Leaf gas exchange measurements

Light saturated photosynthesis

Leaf gas exchange was recorded on plants between 3 and 6 h after treatment (1200–1600 h.) using an open gas exchange system coupled to an infrared gas analyser (LI-6400XT; LI-COR, Inc., Lincoln, NE, USA; Fig. 1). All measurements were made on fully expanded leaves. Light saturated net photosynthesis (A_C) and transpiration (E) were measured using a red and blue light source (6400-40 LI-COR, Inc.) with 2 cm^2 leaf area. All measurements were conducted at constant CO_2 (400 μmol CO_2 mol air^{-1}), constant temperature (25–26 °C) and relative humidity (20–40%). Photosynthetically active radiation was maintained at 2000 μmol photons $m^{-2} s^{-1}$, matching the light intensity commonly observed in the field (37°16 10.49″ N/113°16 53.15″ W, UT, USA; Supporting Information Fig. S10).

CO_2 response curves

CO_2 response curves (0, 200, 400, 600, 800, 1000, 1200, 1400, 1600, 1800 μmol CO_2 mol air^{-1}) were performed on three plants per treatment at 2000 μmol photons $m^{-2} s^{-1}$, 25–26 °C and 20–40% relative humidity. Parameters obtained were stomatal conductance (g_s, mol $m^{-2} s^{-1}$), transpiration rate (E, mmol $m^{-2} s^{-1}$), intracellular concentration (Ci, μmol $m^{-2} s^{-1}$) and net CO_2 assimilation rate (A, μmol $m^{-2} s^{-1}$) according to Farquhar *et al.* (1980), Sharkey (1985) and Sharkey *et al.* (2007). For practical reasons, we distinguished two types of analysis (shown in Supporting Information Figs S2 and S5–S7): analysis of A_C/C_i curves and analysis of stomatal responses (E/gs ratio as a function of increasing CO_2).

Metabolite analysis

Phytohormone analysis was performed by liquid chromatography coupled to a tandem triple quadrupole mass spectrometer (EVO-Q, Bruker Daltonik GmbH, Germany). Frozen leaf lamina tissue was ground to a fine powder and homogenized and aliquoted into 96-well BioTubes (Arctic White LLC). Phytohormones were extracted from plant tissues with (1) ethyl acetate (Bonaventure *et al.*, 2011) and (2) with acidified aqueous methanol followed by two solid-phase extraction (SPE) steps as described in Schafer *et al.* (2015a), with modifications to allow for the recovery of methanol soluble acidic phytohormones. Ethyl acetate extraction was specifically used to extract OPDA, JAs, salicylic acid (SA) and ABA metabolites. SPE extraction was used for the analysis of CKs, JAs, SA and ABA. To recover JAs, SA and ABA from the SPE extraction system, we slightly change the elution of the second SPE step as follows: the samples loaded on the Multi 96 HR-XC column (96 × 25 mg; Macherey Nagel) were washed with 1 mL 1 N HCOOH, and negatively charged phytohormones (JAs, SA and ABA) were eluted with 1 mL of 0.2 N HCOOH in 80% MeOH. Fifty microliter aliquots of this fraction were used for MS analysis. CK-free bases and glucosides were then treated as described previously (Schafer *et al.*, 2015a), eluted with 1 mL 0.35 N NH_4OH in 60% (v/v) MeOH, dried with compressed air and reconstituted in 50 μL 0.1% (v/v) acetic acid for MS analysis.

Chromatographic separation was performed with a BRUKER Advance UHPLC system equipped with a Zorbax Eclipse XDB-C_{18} (50 × 3 mm, 1.8 μm) column (Agilent Technologies). The mobile phase consisted of solvent A (0.05% formic acid and 0.1% acetonitrile in water) and solvent B (MeOH) with the elution gradient profile for the analysis of OPDA, JA, SA and ABA (Time-min/%B): 0/5, 0.5/5, 0.6/50, 2.5/100, 3.5/100, 3.55/5, 4.5/5 at a constant flow rate of

400 µL min^{-1}; and for the analysis of CKs: 0/5, 0.5/5, 0.7/15, 3.5/25, 6.5/70, 6.7/100, 7.7/100, 7.8/5, 8.8/5 at a constant flow of 500 µL min^{-1}. The column was maintained at 42 °C. The UHPLC was coupled to a Bruker EVOQ equipped with a heated electrospray ionization ion source. The mass spectrometer was operated in multi-reaction-monitoring modus as described in Schafer *et al.* (2016). Data acquisition and processing were performed using 'MS data Review' software of the 'Bruker MS Workstation' (Version: 8.2).

Chemicals and standards

Ethyl acetate and methanol for metabolite analysis were purchased from Merck, ammonium hydroxide from Sigma, acetonitrile from VWR, acetic acid from Carl Roth and formic acid (HCOOH) for chromatography from Fisher Scientific. Standards used for identification and normalization of MS measurements were obtained from HPC Standards GmbH (^{13}C-JA-Ile, D_6-JA) and OlChemlm Ltd (D_4-SA, D_6-ABA and CK standards: tZ, tZR, tZROG, tZ7G, cZ, cZR, cZROG, cZ9G, IP and isopentenyl adenine riboside).

Statistical analysis

Data analysis was performed using R-studio (Version 0.99.467–© 2009–2015 RStudio, Inc.) and SPSS (IBM). Quantification of hormones and measurements of photosynthetic parameters were analysed by two-way analyses of variance (ANOVAs) followed by Tukey-honest significant difference test and confirmed with independent Student's *t*-test. The A_C/C_i analysis was performed as described in Sharkey *et al.* (2007). Rubisco activity was additionally analysed with a general linear model; slope and multiple R-squared ($_m r^2$) are provided in the figures. Differences between treatments of carbon assimilation and E/g_s ratio analysis (E/g_s) were analysed by two-way MANOVA models. Linear models were fitted and slope and adjusted R-squared ($_A r^2$) were provided in the figures. Normality of samples was analysed by Shapiro–Wilk test and homoscedasticity with Levene's test. Square root or logarithmic transformations were performed if the tests of the homogeneity of variances were not satisfied.

RESULTS

OS-elicitation decreases A_C in local and systemic leaves

Studies of leaf-level gas exchange in response to natural herbivore attack are difficult to interpret and compare because of differences in spatial and temporal dynamics of tissue loss and vascular damage among replicates. To avoid these problems, we used an elicitation procedure in which standardized puncture wounds were treated either with water ('wounding treatment') or with *M. sexta* oral secretion ('OS-elicitation') so that the wound response could be standardized, separated from the herbivore-specific responses, and precisely timed (Fig. 1b). We conducted leaf-gas exchange measurements of treated (local) leaves and vascular connected undamaged (systemic) leaves (Fig. 1b) 4 h after elicitation.

Consistent with previous reports of herbivore feeding on *N. attenuata* (Halitschke *et al.*, 2011), we observed a significant decrease in light saturated photosynthesis A_C after OS-elicitation in both local ($P = 0.008$) and systemic leaves ($P = 0.008$; Fig. 2, CO_2 assimilation, black boxes). In contrast, the wounding treatment showed only a significant decrease in local leaves ($P = 0.049$), but not in systemic leaves ($P = 0.588$; Fig. 2, CO_2 assimilation, blue boxes). This observation suggests that oral secretion-specific responses were elicited in systemic leaves.

Wounding and OS-elicitation treatments decrease g_s, but not in Rubisco or RuBP turnover

The decrease in A_C coincided with changes in stomatal conductance (g_s, $P = 0.066$; Fig. 2) and transpiration (E, $P = 0.006$), but without influencing intracellular CO_2 concentrations (C_i, $P = 0.093$; Supporting Information Fig. S1). The decrease in g_s may have reduced the flux of CO_2 supplied to the carboxylation centre, decreasing CO_2 assimilation; however these changes were not mirrored in lower C_i values. Other mechanisms have been proposed to drive photosynthetic responses to herbivore attack (Giri *et al.*, 2006), such as (1) Rubisco carboxylation efficiency and (2) RuBP turnover (Fig. 1a). Potential changes in these parameters were evaluated with A_C/C_i curves conducted with local and systemic leaves 3–6 h after elicitation. Rubisco carboxylation efficiency and RuBP turnover were not altered in either local or systemic leaves by either wounding or OS-elicitation treatments (Supporting Information Fig. S2a). Moreover, A_C values, at atmospheric CO_2, were located in the linear range of the A_C/C_i curve, where assimilation is limited by Rubisco carboxylation and CO_2 availability (Farquhar and Sharkey, 1982). The lack of changes in carboxylation efficiency indicates that the decrease in A_C is likely driven by a reduction in the levels of CO_2 as a consequence of decreases in g_s, which is consistent with the decrease in the E/g_s ratio. This could be observed locally after wounding and both locally and systemically after OS-elicitation (Supporting Information Fig. S2b). However, the lack of changes in C_i levels raises the question whether if the limitation of CO_2 by g_s is primarily responsible for the decreases in A_C. Moreover, a systemic reduction was only observed after OS-elicitation, confirming that the response was specific to herbivore perception.

Oral secretion-elicited photosynthetic responses do not differ between wild and laboratory *M. sexta* larvae

A recent report by Barron-Gafford *et al.* (2012) identified differences in the A_C of *Datura* plants in response to the attack from laboratory and wild *M. sexta* larvae. The feeding behaviour of laboratory larvae did not reduce A_C, while that of wild larvae did. We therefore tested if the oral secretion

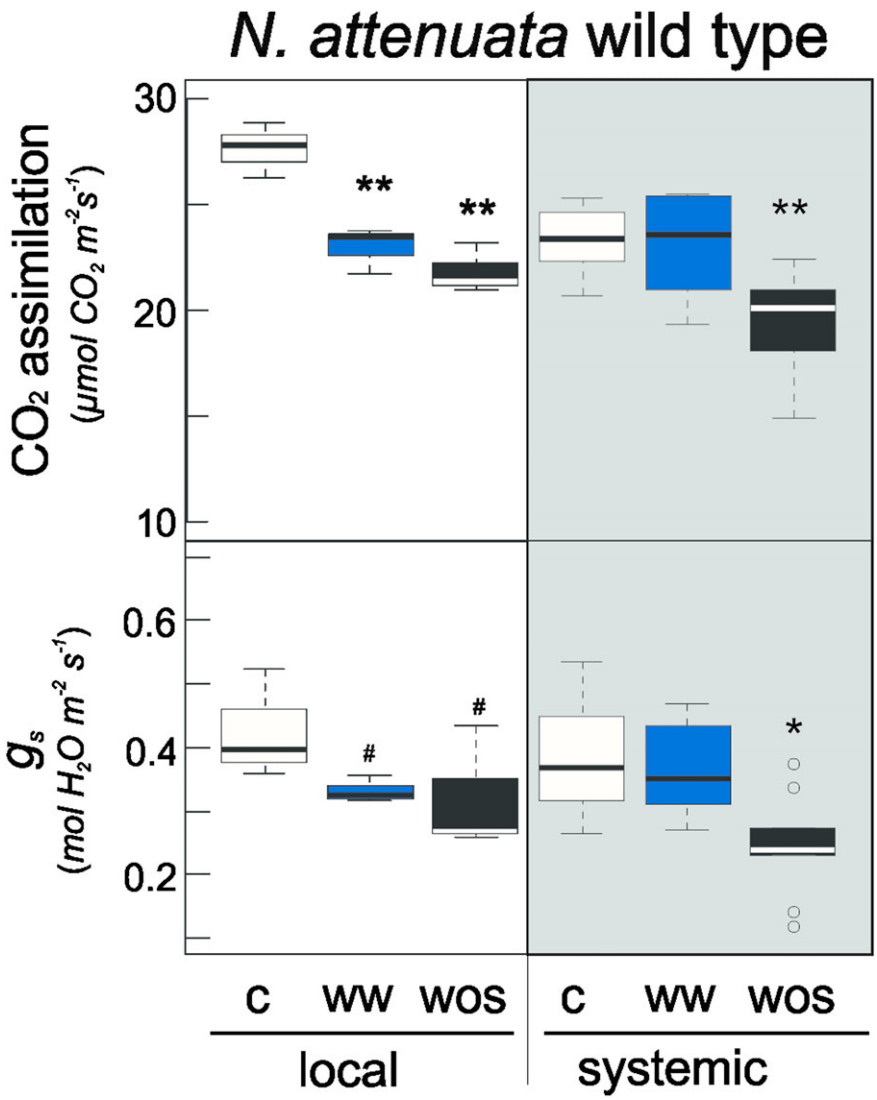

Figure 2. Wounding and oral secretion elicitation decreases carbon assimilation and stomatal conductance in local and systemic leaves. Carbon assimilation and stomatal conductance to water vapour (g_s) rates measured at 400 p.p.m. CO_2 of local and systemic leaves of plants 4 h after wounding (WW, blue boxes) or *Manduca sexta* oral secretion elicitation (WOS, black boxes) and untreated control (WOS, white boxes). Significant differences were analysed by two-way ANOVA. Asterisks indicate significant differences between control and treated samples (Tukey's HSD post-hoc test, $^{**}p < 0.01$, $^{*}p < 0.05$, $\#p < 0.1$). [Colour figure can be viewed at wileyonlinelibrary.com]

of laboratory and wild *M. sexta* populations elicited different responses in photosynthesis and found no significant differences, either in A_C (Supporting Information Fig. S3a) or in the Rubisco-carboxylation efficiency (Supporting Information Fig. S3b). From these results, we infer that oral secretion-elicited photosynthetic responses are conserved among *M. sexta* larvae reared under different dietary regimes.

Known stomatal regulators, JA, ABA and CK, are increased by OS-elicitation

Phytohormones are important regulators of responses to herbivory (Erb *et al.*, 2012), and many are also known to modulate photosynthesis and stomatal behaviour (Acharya and Assmann, 2009). Herbivore-specific phytohormone regulators are known to accumulate minutes to hours after herbivore elicitation and to signal and induce changes that last from hours to days. We harvested leaves 1 h after wounding and OS-elicitation from an experiment run in parallel to the photosynthetic analysis and quantified changes in phytohormones (Fig. 1b). We found significant changes in the levels of JAs, SA, CKs and ABA, all known to regulate stomatal behaviour and to respond to OS-elicitation in both local and systemic tissues; the results were (Fig. 3a and Supporting Information Fig. S4) consistent with previous reports (Halitschke *et al.*, 2001; Dinh *et al.*, 2013; Schafer *et al.*, 2015a).

Jasmonic acids, such as OPDA, JA and JA-Ile, were up-regulated after wounding, and these wound-induced responses were amplified by oral secretions (Fig. 3a and Supporting Information Fig. S4). An important interaction partner of JAs is

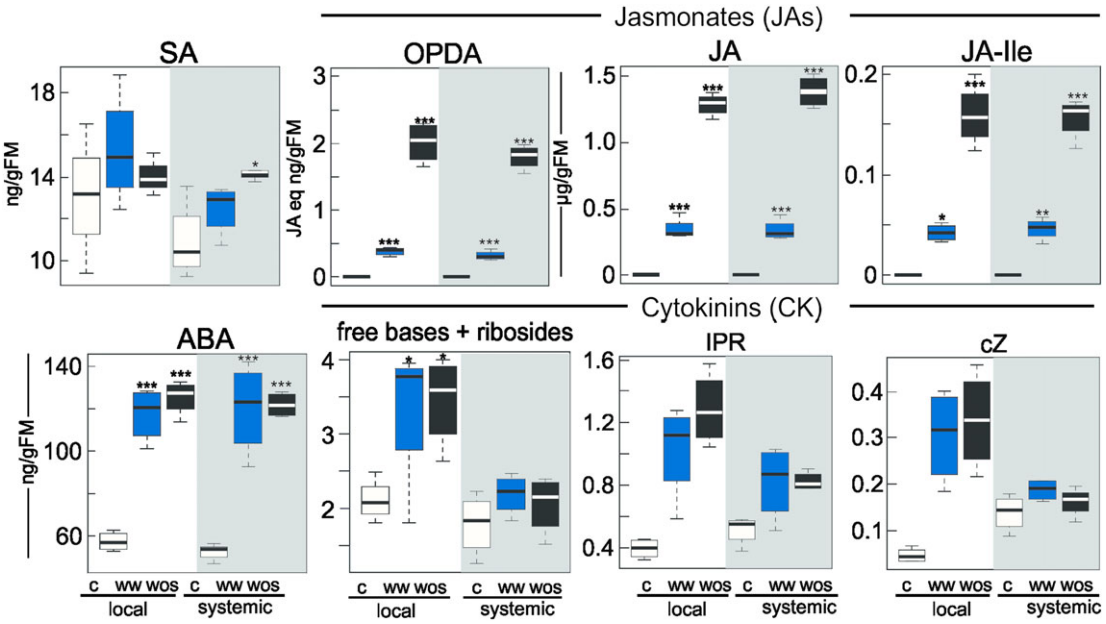

Figure 3. Accumulation of plant hormones involved in stomatal responses induced by wounding and oral secretions. Levels of abscisic acid (ABA), salicylic acid (SA), active cytokinins (CK; isopentenyl adenine, *trans*-zeatin, *cis*-zeatin and their respective ribosides) and jasmonates (JAs: 12-oxo phytodienoic acid, OPDA; Jasmonic acid, JA; JA-isoleucine conjugate, JA-Ile) on leaves of *Nicotiana attenuata* plants 1 h after treatment. Plants were treated with wounding (WW, blue boxes) or *Manduca sexta* oral secretion elicitation (WOS, black boxes), and untreated plants were used as controls (WOS, white boxes). Significant differences were analysed by two-way ANOVA. Asterisks indicate significant differences between control and treated samples (Tukey's HSD post hoc test, ***$p < 0.001$, **$p < 0.001$, *$p < 0.05$). JA eq, jasmonic acid equivalents; FM, fresh mass. [Colour figure can be viewed at wileyonlinelibrary.com]

SA. Levels of SA were found only to increase in systemic leaves after OS-elicitation (Fig. 3a) and hence could account for the differences in photosynthetic responses. Both JA and SA signalling promote stomatal closure (Acharya and Assmann, 2009), but their roles in regulating g_s are far from clear. However, they both are thought to act in concert with ABA (Daszkowska-Golec *et al.*, 2013).

Levels of ABA increased twofold after both wounding and OS-elicitation treatments in local and systemic tissues (Fig. 3a). ABA triggers stomatal closure decreasing stomatal conductance in response to different types of biotic and abiotic stresses (Daszkowska-Golec *et al.*, 2013). Hence, increases in ABA could also account for the elicited g_s decreases.

Stomatal responses to CKs are less clear; CKs promote stomatal opening at physiological levels as well as stomatal closure at high concentrations (Acharya and Assmann, 2009). However, the direct activity of CK signalling in stomatal closure is not known, but the inhibition of ABA-induced stomatal closure by CKs was recently described (Nguyen *et al.*, 2016). The overall levels of the CK base and ribosides pools increased locally after wounding and OS-elicitation (Fig. 3a), although isopentenyl adenine riboside and *cis*-zeatin riboside (cZR) increased in both, local and systemic, tissues (Fig. 3a). The accumulation of *trans*-zeatin CK types (tZ, tZR) was not changed by treatments, but levels were higher in systemic tissues than in local tissues, a result consistent with higher levels typically found in younger leaves, a general trend for CKs (Ori *et al.*, 1999). Changes in CK levels were therefore not correlated with changes in photosynthesis, but the possibility that CK signalling might interact with ABA in a feedback regulation cannot be ruled out.

Disrupting stomatal responses to ABA signalling by silencing MPK4 in shoots impairs g_s responses to OS-elicitation in systemic but not in local leaves

The transgenic line irMPK4 was used to test if stomatal closure after OS-elicitation could be regulated by ABA signalling, because irMPK4 plants are impaired in stomatal responses to ABA signalling (Hettenhausen *et al.*, 2012). ABA could function directly by changing local (1) and systemic stomatal behaviour (2) or (3) indirectly by changing responses in roots (Fig. 4a; McAdam *et al.*, 2016). We examined these hypotheses with experiments using irMPK4 plants and grafts of WT shoots to irMPK4 roots (WT/irMPK4). In contrast to WT, irMPK4 plants showed no significant decrease in A_C or g_s, after wounding in local leaves and OS-elicitation in systemic leaves (Fig. 4b and Supporting Information Fig. S5). In contrast, OS-elicitation of irMPK4 lines reduced g_s ($P = 0.020$), and A_C ($P = 0.026$) significantly in local leaves. Interestingly, this response occurred despite the impairment of irMPK4 plants to ABA perception and therefore the lack of changes in the E/g_s ratio (Supporting Information Fig. S5b). From these results, we infer that MPK4 and ABA signalling regulate responses to wounding in local tissues and responses to OS-elicitation in systemic tissues, but not after OS-elicitation in local tissues, highlighting that not only do the photosynthetic responses differ between wounding and OS-elicitation but their regulation after OS-elicitation also differs between local and systemic leaves. In addition, the carboxylation efficiency of Rubisco and RuBP turnover in irMPK4, as well as in WT plants, was not influenced by wounding and OS-elicitation in

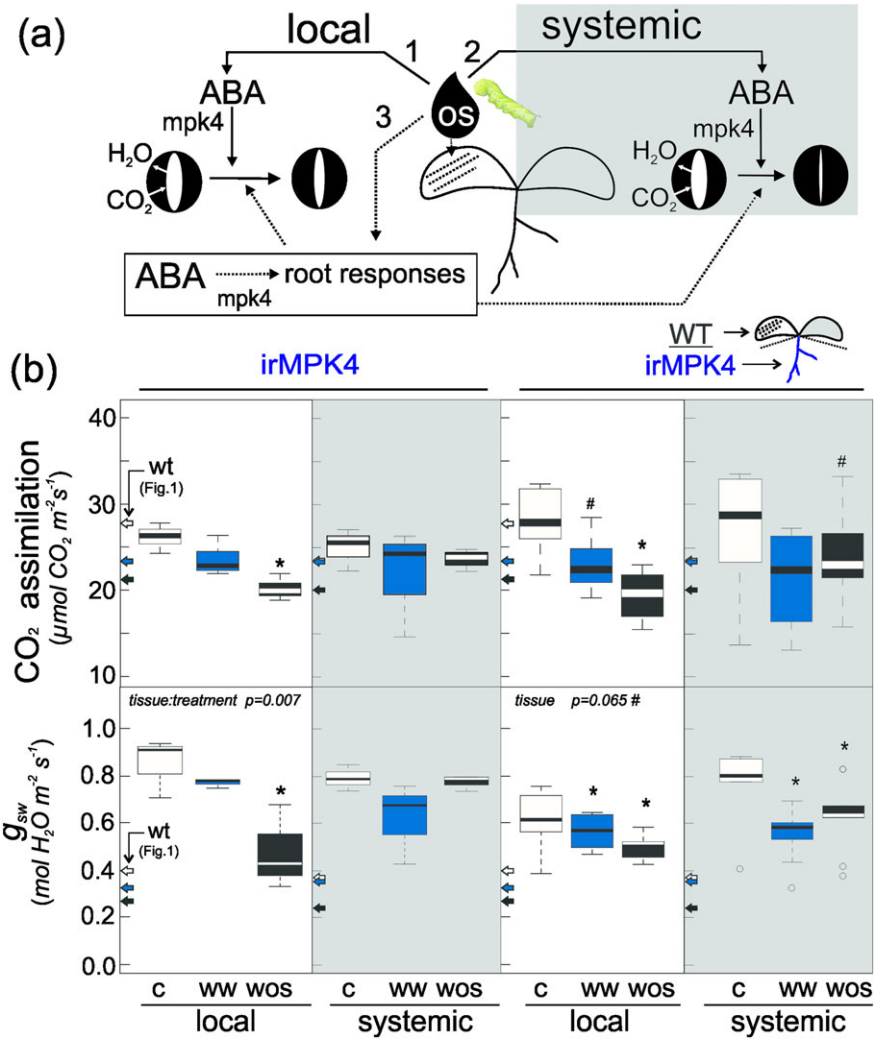

Figure 4. Mitogen-activated protein kinase-4 (MPK4) signalling is required for responses in systemic leaves induced by oral secretion (OS) and in local leaves for responses to wounding but not to OS. (a) Scheme of three hypothetical alternative pathways for the regulation of stomatal responses by abscisic acid (ABA) through the MPK4 in local and systemic leaves and roots induced by wounding and OS-elicitation. Wounding (dashed lines on local leaf) and perception of OS elicits the accumulation of the stomatal regulator ABA in local and systemic leaves. The increased levels of ABA in local (1) and systemic (2) leaves could lead to direct changes in stomatal conductance (g_s). Alternatively, changes in g_s might result from ABA signalling in roots (3). Solid arrows display responses on the basis of the literature and the metabolomic analysis. Dashed arrows display hypothetical responses of the known activity of ABA. (b) Photosynthetic responses were measured at 400 p.p.m. of CO_2 in leaves of transgenic *Nicotiana attenuata* plants silenced for NaMPK4 gene (irMPK4) and of WT/irMPK4 (shoot/root) grafted plants after wounding (WW, blue boxes) or *Manduca sexta* OS-elicitation (WOS, black boxes) and of untreated control (WOS). For comparison, the averages of the responses observed in the wild type (WT) plants (Fig. 1) are indicated by arrows (the same colour code) along the Y-axis. Significant differences were analysed by two-way ANOVA. Asterisks indicate significant differences between control and treated samples (Tukey's HSD post hoc test, $*p < 0.05$, $\#p < 0.1$). [Colour figure can be viewed at wileyonlinelibrary.com]

local and systemic tissues as compared with non-treated plants (Supporting Information Fig. S5a).

The response in photosynthesis in local tissues observed in WT/irMPK4 grafts at atmospheric CO_2 did not differ from those of WT plants (Fig. 4b). This suggests that local responses regulated through NaMPK4 are mainly based in the shoot and not regulated by root MPK4 expression. However, in systemic leaves, clear differences were observed in WT/irMPK4 plants after wounding (g_s, $P = 0.026$), but not after OS-elicitation compared with WT. In addition, the E/g_s ratio revealed an increase in the range of responses to atmospheric CO_2 in the WT/irMPK4 plants (Supporting Information Fig. S5b), suggesting a regulatory role of MPK4 in roots on water flux that likely explains the changes observed in g_s of systemic leaves.

Cytokinin perception regulates g_s after wounding locally and after OS-elicitation systemically

Cytokinin (base and ribosides) levels were higher after OS-elicitation compared with wounding in local leaves (Fig. 3). We examined the role of CK signalling in photosynthetic responses after herbivore perception using a transgenic line

silenced in the CK receptors, NaCHK2 and 3 (irCHK2/3) and its interaction with ABA-MPK4 signalling using a hemizygous cross of irCHK2/3 with irMPK4 (irCHK2/3*irMPK4) in which both ABA and CK signalling were silenced (Fig. 5a).

Plants impaired in CK signalling displayed a similar response pattern as WT, but with no significant differences in A_C or g_s after either wounding or OS-elicitation treatments locally and systemically. The A_C of local leaves ($P = 0.057$) and the g_s of systemic leaves ($P = 0.085$) tended to be lower after OS-elicitation of irCHK2/3. However, differences among treatments and tissues were clearly in the E/g_s ratio analysis; here, the slope of the E/g_s ratio was found to increase after wounding and OS-elicitation in local leaves of irCHK2/3, a response not observed with WT or irMPK4 plants (Supporting Information Figs S2b and S5b). Together, these data suggest that CKs modulate the strength of the responses and likely recruit changes in other physiological process, such as hydraulic conductivity.

Interestingly, silencing NaCHK2/3 and NaMPK4 together (irCHK2/3*irMPK4) revealed a phtosynthetic response similar to that of WT plants (Fig. 5b). In spite of the impaired responses to ABA in the irMPK4 plants, the irCHK2/3*irMPK4 crosses displayed a decrease in A_C and g_s in local leaves after wounding and in systemic leaves after OS-elicitation. These data indicate that the lack of decrease in A_C and g_s after wounding and OS-elicitation in local and systemic leaves in irMPK4 plants (Fig. 4b), which is likely due to inhibition of ABA signalling, is also mediated through CK signalling as a feedback signal that modulates stomatal responses. An alternative explanation would be that the silencing efficiency in the hemizygous irCHK2/3*irMPK4 plants was lower than that of the homozygous crosses; however, other related

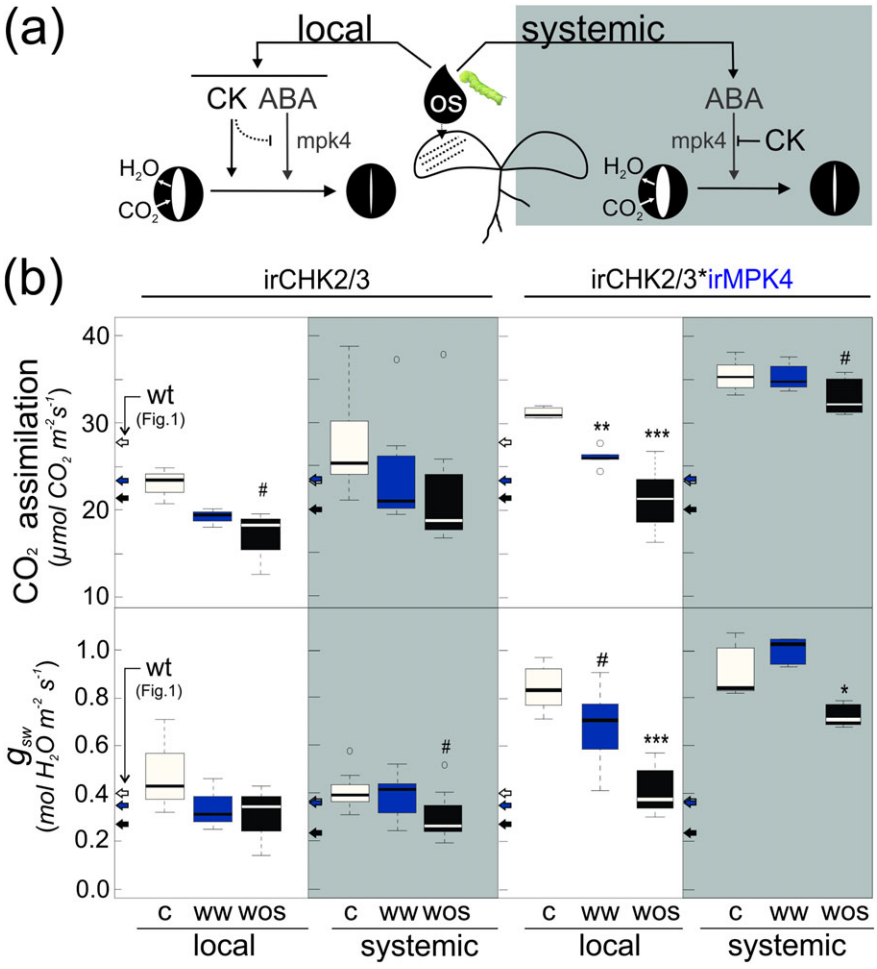

Figure 5. Impaired abscisic acid (ABA) signalling and cytokinin (CK) perception suggest a third unknown regulator of stomatal responses induced by oral secretion (OS) elicitation. (a) Scheme of stomatal responses to the possible interaction among ABA and CKs in local and systemic leaves after OS-elicitation. Wounding and perception of herbivore OS induces the accumulation of ABA and CK in local and ABA in systemic leaves. (b) Leaf gas exchange responses measured at 400.p.p.m. CO_2 of *Nicotiana attenuata* CK chase-domain containing his kinase receptors 2 and 3 (irCHK2/3) and crosses of irCHK2/3 with irMPK4 (irMPK4*irCHK2/3) after wounding (WW, blue boxes) or *Manduca sexta* OS-elicitation (WOS, black boxes) and of untreated control (WOS) leaves from untreated plants. For comparison, the averages of the responses observed in the wild type (WT) plants (Fig. 1) are indicated by arrows (the same colour code) along the Y-axis. Significant differences were analysed by two-way ANOVAs. Asterisks indicate significant differences between control and treated samples (Tukey's HSD post hoc test, ***$p < 0.001$, **$p < 0.001$, *$p < 0.05$, #$p < 0.1$). [Colour figure can be viewed at wileyonlinelibrary.com]

phenotypes described previously in irCHK2/3 and irMPK4 homozygous plants were clearly observed in the irCHK2/3*irMPK4 crosses, such as the distinctive phylotaxis with increased branching and premature leaf senescence of irCHK2/3 plants, and the greener leaves and greater flower number in the early stages of flowering in irMPK4 plants. From these results, we infer that silencing efficiency of the hemizygous crosses was comparable with that of the homozygous plants, as has been the case in previous work with hemizygous crosses (Onkokesung *et al.*, 2010; Hettenhausen *et al.*, 2013; Heinrich *et al.*, 2013).

In addition, the lack of stomatal response to increasing levels of CO_2 observed in irMPK4 lines (Supporting Information Fig. S5b) was not observed in irCHK2/3*irMPK4 (Supporting Information Fig. S6b). The changes in the slopes of the E/g_s ratio observed in the CK-impaired lines were enhanced in the irCHK2/3*irMPK4 plants. Local and systemic leaves of irCHK2/3*irMPK4 plants displayed an increased rate of E to changes in g_s induced by OS-elicitation. This indicates a role for CK perception in the regulation of stomatal responses to changes in CO_2. In addition, in this line (irCHK2/3*irMPK4), we also found a significant decrease in the levels of C_i in local ($P = 0.047$) and systemic ($P = 0.033$) tissues after OS-elicitation, a result consistent with changes in mesophyll conductance (Supporting Information Fig. S8).

OPDA regulates oral-secretion-elicited g_s and A_C responses in local leaves, but JA/JA-Ile regulates responses in systemic leaves

The aforementioned results highlighted the need for an additional signal to regulate specific photosynthetic responses to OS-elicitation in local leaves. One class of hormones specifically enhanced after OS-elicitation are the JAs (Stitz *et al.*, 2011). In *Arabidopsis*, external application of a homolog of JA-Ile, coronatine, leads to changes in g_s (Attaran *et al.*, 2014), and recently, OPDA was found to induce stomatal closure in response to drought (Savchenko *et al.*, 2014). We therefore explored the role of JAs in the regulation of oral secretion-elicited photosynthetic responses (Fig. 6 and Supporting Information Fig. S7).

We used two transgenic lines that were silenced either upstream or downstream of OPDA biosynthesis, namely, irAOC and irACX1, respectively (Kallenbach *et al.*, 2012). Silencing AOC resulted in the loss of all photosynthetic responses observed in WT (Fig. 6). These data imply that

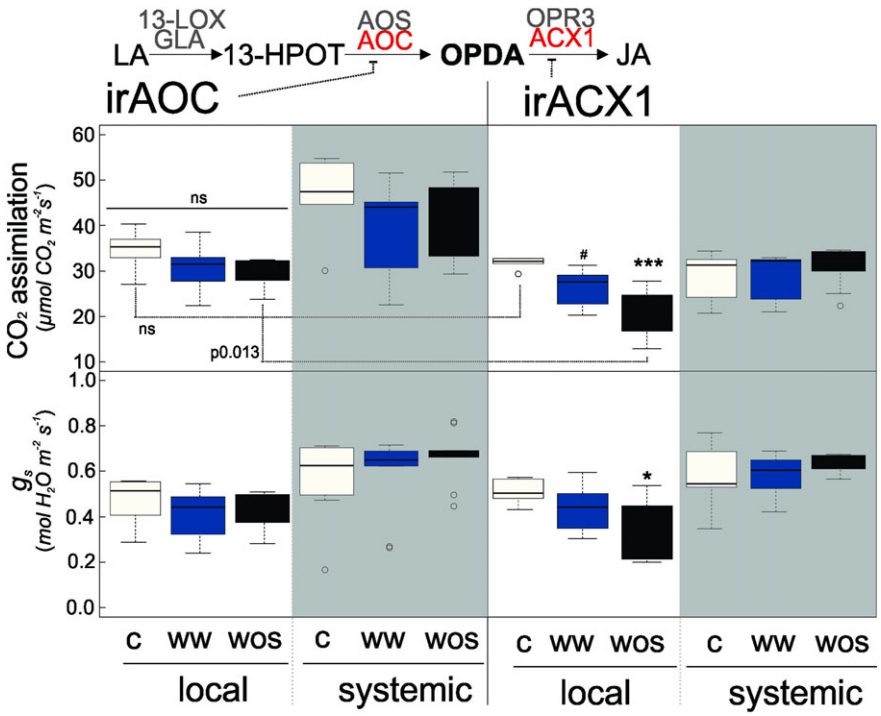

Figure 6. 12-oxo phytodienoic acid regulates stomatal responses to wounding and herbivore elicitation in local leaves but systemic responses require JA/JA-Ile signalling. Scheme of the biosynthetic pathway for 12-oxo phytodienoic (OPDA) acid and jasmonic acid (JA) (top of figure). Polyunsaturated fatty acids [i.e. linolenic acid (LA)] are metabolized by glycerolipases (GLA) and lipoxygenases (13-LOX) to 13(S)-hydroperoxy-octadecatrienoic acid (13-HPOT). 13-HPOT is cyclized by allene oxide cyclase (AOC) and oxidized by allene oxide synthase (AOS) to OPDA, which undergoes a set of REDOX reactions involving OPDA-reductase (OPR3) and acyl-CoA transferase-1 (ACX1) to form JA. Box plots show the carbon assimilation and stomatal conductance to water vapour (g_s) rates measured at 400 p.p.m. CO2 in local and systemic leaves of *Nicotiana attenuata* transgenic lines (irAOC and irACX1) 4 h after wounding (WW, blue boxes) or *Manduca sexta* oral secretion elicitation (WOS, black boxes) treatments and of untreated control (WOS) plants. Significant differences were evaluated by MANOVA. Important non-significant (ns) and significant p-values are displayed. Asterisks indicate significant differences between control and treated samples analysed by Tukey's HSD test (***$p < 0.001$, **$p < 0.001$, *$p < 0.05$, #$p < 0.1$). [Colour figure can be viewed at wileyonlinelibrary.com]

JAs are required for oral secretion-elicited changes in g_s and A_C. However, plants silenced in JA biosynthesis downstream of OPDA (irACX1) displayed largely WT responses to OS-elicitation. We found decreases in g_s ($P = 0.023$) and A_C ($P = 0.000$) in local tissues after OS-elicitation, and additionally in Ac ($P = 0.049$) in systemic leaves. In contrast to the WT responses, OS-elicitation of irACX1 plants did not result in g_s and A_C responses in systemic leaves. From the restoration of the response in g_s and A_C in local leaves in irACX1 plants compared with those of AOC plants, we infer that OPDA regulates photosynthetic gas exchange responses locally, while oral secretion-induced photosynthetic responses in systemic leaves required JA or JA-Ile signalling.

DISCUSSION

Insect attack clearly influences photosynthetic responses in very different ways, and some of these differences result from the modes in which insects from different feeding guilds damage plants (Baldwin, 1990). With this study, we enhance our understanding of the mechanisms involved in these herbivore-specific responses. We analysed how wounding and the perception of herbivore-associated elicitors evoke photosynthetic changes in local and systemic leaves of *N. attenuata* plants and identified the phytohormone signalling pathways regulating these responses (Fig. 7). Our results revealed a specific and significant decrease in A_C after the perception of herbivore

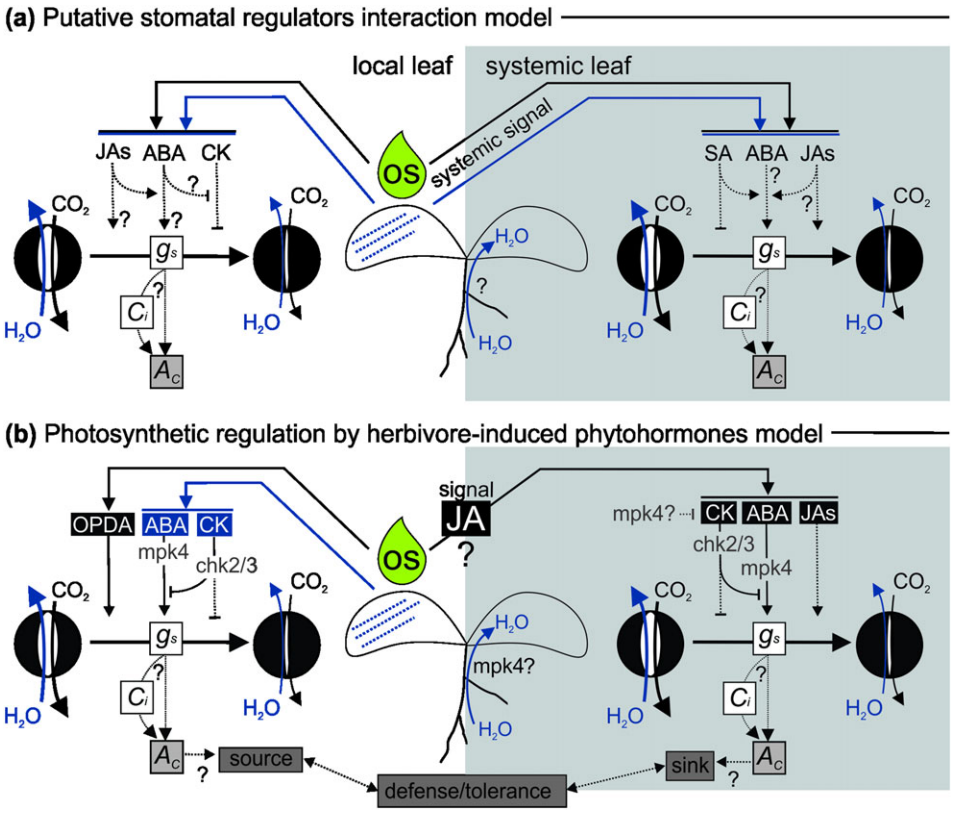

Figure 7. Hypothetical framework (a) and working - results summary - model (b) of the photosynthetic regulation by herbivore-induced phytohormones. (a) Hypothetical framework: model for the putative involvement and interaction of herbivore-induced phytohormones (Fig. 2) in the regulation of stomatal conductance (g_s) and carbon assimilation (A_C) (Fig 1). The induction of phytormones (long solid arrows) by wounding (dotted-lines on leaf; blue arrow) or *Manduca sexta* OS (black arow) in local damaged (white box) and systemic un-damaged leaves (grey box) signals stomatal responses in leaves modifying g_s. Changes in g_s contribute to changes in A_C by either temporarily changing intercellular CO_2 levels (C_i) or other related photosynthetic unknown processes. Alternatively, stomatal responses can be changed indirectly by shifting physiological root responses (local leaf to root arrow) affecting water fluxes. Dash arrows represent possible unknown processes supported by the literature. Question marks highlight unknown responses and processes analysed in this study. (b) Working model: perception of herbivore elicitors decreases g_s and A_C through OPDA in damaged local leaves, while a complex interaction between ABA, CK and JA signalling pathways mediate g_s and A_C responses after wounding and OS-elicitation in local and systemic leaves respectively. Wounding and the perception of herbivore OS cues induced changes in g_s and A_C of local and systemic leaves. Local changes in g_s to OS elicitation are regulated by OPDA (Fig. 6), while responses in systemic leaves are regulated by ABA signalling through MPK4 (Fig.4) interacting with CK signalling via receptors CHK2/3 (Fig. 5) and JA signalling (Fig.6). Local changes of stomatal conductance to wounding are also mediated by ABA-MPK4, CK-CHK2/3 and likely OPDA signalling (Figs 4–6). These changes in stomatal conductance may induced transitory changes in C_i or other photosynthetic related unknown processes leading to a decrease in A_C. Changes in A_C could then affect leaf source-sink relations and defence/tolerance responses. In roots, MPk4 signalling also regulates water balance affecting gs (Fig. 4), likely through changes hydraulic conductivity induced by ABA. JAs, jasmonates/jasmonic acid; OPDA, 12-oxo-phytodenoic acid; ABA, absisic acid, CK, cytokinins; SA, salicylic acid; MPK4, mitogen-activated protein kinase-4; CHK2/3, chase-domain containing his kinase 2 and 3; OS, oral secretion. [Colour figure can be viewed at wileyonlinelibrary.com]

elicitors in local and systemic leaves. Interestingly, this decrease co-occurred with changes in g_s, but not in Rubisco carboxylation or RuBP turnover. These changes in g_s were found to be locally regulated by OPDA and systemically by a suite of interactions among JAs, ABA and CK. Photosynthetic responses have often been described as a consequence of the damage resulting from herbivore feeding rather than an active process elicited in plants in response to perceiving the attack by herbivores. Here, we show that *N. attenuata* plants actively modulate photosynthesis after perceiving herbivore elicitors. This modulation may precede slower biochemical and metabolic reconfigurations that are thought to be responsible for the changes in the patterns of senescence and photosynthesis in the unattacked leaves of herbivore-attacked plants (Rosenthal and Kotanen, 1994; Baldwin and Ohnmeiss, 1994).

Early changes in A_C and g_s in response to perception of herbivore cues

Decreases in A_C after herbivory have been suggested to occur as a result of changes in Rubisco carboxylation and RuBP turnover (Fig. 1; Peterson, 2000; Thomson *et al.*, 2003; Giri *et al.*, 2006; Bilgin *et al.*, 2010). However, most studies have assessed photosynthetic changes 24 h after herbivore attack, a time when changes in photosynthesis are likely influenced by other induced responses such as changes in sugar and secondary metabolite accumulations. We analysed photosynthetic responses 4 h after wounding and OS-elicitation and found no significant changes in Rubisco carboxylation and RuBP turnover in any treatment or tissue in WT plants (Fig. 2). Metabolic reconfigurations are not expected to be fully established 4 h after herbivore elicitation, and we infer that other mechanisms regulate short-term responses in photosynthesis triggered by herbivore elicitors perception.

We observed a decrease in g_s, simultaneously with a reduction in A_C, for all treatments that affected photosynthesis in all the genotypes analysed (Figs 2 and 4–6). These changes in g_s and A_C at atmospheric levels of CO_2 fall into the linear (Rubisco activity) increase of the A_C/C_i curves, where the rate of carboxylation is thought to be limited by Rubisco activity and sub-stomatal CO_2 concentrations (Supporting Information Fig. S2), indicating that decreases in g_S may reduce substomatal CO_2 concentration at the carboxylation site and thereby limit A_C. However, no changes were observed in C_i levels (Fig. S1). The lack of change in C_i levels could be explained by a reduction in mesophyll conductance, leading to decrease chloroplast CO_2 concentrations (C_C) levels and therefore reducing the rate of net CO_2 fixation. C_i levels could also be temporarily reduced moments after treatment, before the observed changes in photosynthesis (3–6 h after treatment), leading to increases in Rubisco oxygenation. A temporary increase in the rate of Rubisco oxygenation could result in a surge in photorespiratory hydrogen peroxide, or an ROS burst. This ROS burst could serve as a redox-based signalling event that elicits adjustments in the photosynthetic apparatus (i.e. reducing Rubisco molecules) and therefore A_C without affecting the efficiency of carboxylation. C_i levels may regain quilibrium, but inactivation or decreases in Rubisco may continue by the time of the measurements. Alternatively, the change in A_C/C_i relation could be a consequence of patchy stomatal behaviour affecting C_i levels induced by the treatments (Pospíšilová and Šantrůček, 1994); A_C rates measured under heterogeneous stomatal patches behaviour, for a given C_i, are generally lower than A_C rates under homogeneous stomatal behaviour.

While the changes in photosynthesis elicited by herbivore elicitors of piercing insects have been reported (Halitschke *et al.*, 2011), the regulation of stomata and photosynthetic A_C by herbivore elicitors of chewing herbivores, such as in *M. sexta* oral secretions, is a novel observation.

Signalling and regulation of g_s and A_C induced by herbivore elicitation

Prior to this study, little was known about the signalling pathways regulating g_s by herbivore attack. Changes in g_s in response to herbivory have been attributed to indirect effects such as changes in turgor pressure or evapotranspiration as a result of vascular damage (Nabity *et al.*, 2009). Moreover, previous studies have focused on photosynthetic responses to the damage caused by actively feeding herbivores, a treatment that blends different types of elicitors with different kinetics of elicitation and makes the results challenging to interpret. The simulated herbivory treatment used here avoided damage to the major vascular tissues of the lamina and disentangled wounding from herbivore elicitation effects with a precise elicitation kinetic. These procedures allowed us to observe specific differences in g_s between treated leaves of WT and transgenic lines (i.e. irMPK4, Fig. 4) and dissect its regulation.

Regulation of g_s and A_C by JAs, MPK4 and CK signalling after wounding and OS-elicitation

Regulatory mechanisms have been indirectly inferred from transcriptomic and proteomic analyses, but they have not been directly tested. We identified at least two pathways regulating photosynthetic responses, which were differentially regulated by wounding and OS-elicitation: one regulating local responses to wounding and systemic responses after OS-elicitation and the other pathway regulating local responses after OS-elicitation.

OPDA regulates g_s and A_C in local leaves but systemic responses require JA/JA-Ile signalling

We analysed the role of OPDA in the OS-elicited regulation of g_s by silencing jasmonate biosynthetic genes upstream and downstream of OPDA biosynthesis (irAOC and irACX1, Fig. 6). OPDA is an intermediate of JA biosynthesis, but it also functions as signal in plant development and in stomata regulation during drought stress (Dave *et al.*, 2011; Dave and Graham, 2012; Bosch *et al.*, 2014; Savchenko *et al.*, 2014). OPDA was shown to induce stomatal closure and to increase guard cell sensitivity to ABA in response to drought stress in Arabidopsis

(Savchenko *et al.*, 2014). Consistently, our results revealed decreases in g_s in local tissues after wounding and OS-elicitation in irACX1 but not in irAOC plants. However, systemic g_s and *Ac* responses relied on JA/JA-Ile signalling (Fig. 6). Interestingly, a recent report reported a similar pattern (OPDA → local, JA/JA-Ile → systemic) for proteinase inhibitor II-transcript accumulations in tomato after *M. sexta* feeding (Bosch *et al.*, 2014). Similarly, in *N. attenuata*, trypsin protease inhibitor (TPI) activity of irMPK4 plants, which are impaired in their responses to ABA, was correlated with levels of JA (fig. 2 in Hettenhausen *et al.*, 2013) and OPDA after OS-elicitation (Supporting Information Fig. S9) and are not regulated by JA-Ile/COI signalling (Hettenhausen *et al.*, 2013). Interestingly, a correlation between the accumulation of TPIs and decreases in photosynthesis was previously reported in *N. attenuata* 24 h after *M. sexta* herbivory (Nabity *et al.*, 2013). This correlation was also found to depend on JA biosynthesis, as LOX transgenic plants failed to display oral secretion-elicited changes in either TPI or photosynthesis. The observation that the TPI defence was found regulated in tomato and *N. attenuata* in a manner similar to the regulation of the responses in photosynthesis after OS-elicitation underscores the likely crosstalk between defence and photosynthesis regulation, a crosstalk important for the coordination of defence and tolerance responses to herbivore attack.

12-Oxo-phytodienoic acid may decrease g_s either directly or indirectly, by increasing ABA sensitivity. OPDA increases sensitivity to ABA in stomatal responses to drought stress in Arabidopsis, but it can also act independently of ABA signalling (Savchenko *et al.*, 2014). A certain threshold in OPDA levels is likely required to regulate stomatal responses independently of ABA. Such a threshold response could explain why g_s was less affected by wounding and more by OS-elicitation in WT plants (Fig. 2) in which OPDA levels were moderately increased (Fig. 3) and also why in oral secretion-elicited irMPK4 plants, in which OPDA levels are dramatically elevated (Supporting Information Fig. S9), g_s was not affected by ABA (Fig. 4). The fact that g_s was regulated by OPDA instead of ABA-irMPK4 signalling after OS-elicitation suggests a possible functional redundancy between ABA and JAs. Such redundancy and crosstalk in phytohormonal signalling likely enables plasticity in a plant's responses to important fitness-determining environmental constraints. The role of OPDA signalling in herbivore-induced responses has been often underestimated, and here, we show that not only is OPDA an important signal regulating herbivore-induced responses but also its involvement in mediating photosynthetic-gas exchange responses (Fig. 7).

ABA and MPK4 mediate g_s and A_C responses locally after wounding and systemically after OS-elicitation

Abscisic acid is arguably the most important stomatal regulator among phytohormones (Pospisilova, 2003; Kim *et al.*, 2010). ABA promotes stomatal closure by increasing concentration of Ca^{+2} in guard cells, which changes the activity of potassium (K^+) and anion channels leading to an efflux of anions and K^+ from guard cells that result in a loss of turgor pressure and subsequent stomatal closure (Kim *et al.*, 2010). Because guard cells of *N. attenuata* plants silenced in *NaMPK4* expression do not respond to ABA, we used irMPK4 plants to study responses regulated by ABA signalling (Hettenhausen *et al.*, 2012) and found that the oral secretion-elicited decreases in g_s and A_C in systemic leaves required MPK4/ABA signalling but not in local leaves (Fig. 4 and Supporting Information Fig. S5). These unexpected results highlight the differences in the oral secretion-regulated signalling of photosynthetic responses between local and systemic leaves.

Abscisic acid levels are commonly higher in roots, and root ABA signalling is known to influence hydraulic conductivity that in turn influences g_s (Olaetxea *et al.*, 2015). To examine these possible long-distance interactions, we examined the role of MPK4 signalling in roots by micrografting WT shoots to irMPK4 scions. ABA levels were higher in roots, and it is likely that these changes in roots induced by ABA signalling influence hydraulic conductivity, which in turn would affect g_s (Olaetxea *et al.*, 2015). Interestingly, the decrease in g_s and A_C in local tissues in WT/irMPK4 grafts was not different from that of WT plants (Fig. 4 and Supporting Information Fig. S6), suggesting a local regulation of photosynthetic responses in treated leaves. In contrast, the systemic leaves showed a decrease in g_s after wounding (Fig. 4) not observed in WT plants (Fig. 2 and Supporting Information Fig. S2). Apparently, a systemic root signal mediated by MPK4 is required for the modulation of g_s in systemic leaves after wounding. Because A_C levels were not significantly altered in the grafted plants, we speculate that changes in root hydraulic conductance (L_o) resulted in modifications in water flux, an inference consistent with the increased range of responses in the E/g_s analysis of systemic leaves (Supporting Information Fig. S6b). A recent report showed that shoot-to-root signalling decreased L_o after leaf cutting and that the L_o response was mediated by aquaporins in several species (Vandeleur *et al.*, 2014). The expressions of aquaporins are affected after wounding and OS-elicitation in *N. attenuata* plants (Supporting Information Fig. S11; Kim *et al.*, 2011), and future experiments should analyse changes in L_o and its regulation by MPK4 after wounding and OS-elicitation.

CK and ABA crosstalk regulates g_s in systemic leaves after OS-elicitation and to changes in CO_2

We analysed CK and its interactions with MPK4/ABA signalling by using lines impaired in CK perception and NaMPK4: irCHK2/3 and irCHK2/3*irMPK4, respectively. CK are positive regulators of stomatal opening and can inhibit ABA-induced stomatal closure in other plant species (Tanaka *et al.*, 2006). Concentrations of different CK types are changed after wounding and OS-elicitation in *N. attenuata* plants (Fig. 3; Schafer *et al.*, 2015a). However, our results failed to indicate a clear regulation of photosynthesis responses induced by CK perception alone by either treatment (Fig. 5) and only a decrease in the strength of the responses for all treatments, but the response pattern was similar to those of WT plants (Fig. 2).

This is consistent with recent findings showing a decrease in stomatal aperture in lines impaired in CK-signalling in Arabidopsis (Nguyen *et al.*, 2016) and suggest that the main signals responsible for changes in g_s elicited by wounding and OS-elicitation are phytohormones other than CK.

However, an interesting interaction between CK and MPK4/ABA signalling regulating g_s and A_C in local leaves induced by wounding and in systemic leaves by OS-elicitation was observed. This interaction between CK and ABA in the regulation of stomatal responses is well known and understood in molecular terms. CK signalling via AtCHK2/3 receptors is known to block ABA stomatal responses downstream of ABA biosynthesis during drought (Nguyen *et al.*, 2016). MPK4 may inhibit CK signalling, and the putative increased CK signalling by silencing NaMPK4 may have impaired ABA responses in irMPK4 (Hettenhausen *et al.*, 2012), while silencing both NaMPK4 and CK signalling would allow ABA-mediated stomatal closure in irCHK2/3*irMPK4 plants (MPK4┤CK┤ABA). While there are many untested assumptions in this scenario, it is consistent with the fact that plants silenced in CK signalling respond to ABA as WT plants do (Nguyen *et al* 2016) and that ABA mediates g_s responses locally to wounding and systemically to OS-elicitation, as observed in this study.

The interaction between ABA, MPK4 and CK signalling was also apparent from the g_s/E responses to different concentrations of CO_2, and the results are consistent with the proposed model (MPK4┤CK┤ABA). Silencing MPK4 in cultivated tobacco and *N. attenuata* plants impairs stomatal responses to changes in CO_2 (Supporting Information Fig. S5b; Marten *et al.*, 2008; Hettenhausen *et al.*, 2012). Unexpectedly, irCHK2/3*irMPK4 plants were not impaired in their stomatal responses to changes in CO_2 (Supporting Information Fig. S6b). Apparently, silencing CK signalling compensates for the impaired response to CO_2 in NaMPK4-silenced plants. ABA signalling has been shown to regulate at least partially CO_2-induced stomatal closure (Chater *et al.*, 2015) and the current models of hormonal regulation of stomatal responses induced by CO_2 have only considered the effects of ABA (Engineer *et al.*, 2016). The observation that CK signalling interacts with MPK4 in regulating stomatal responses induced by changes in CO_2, suggests that CK regulation and its interaction with ABA in the regulation of g_s should be included in future modelling efforts.

Specific activity of herbivore perception decreasing A_C and g_s in local and systemic leaves

Plants can clearly perceive and distinguish between mechanical wounding and the wounding caused by the feeding behaviour of different feeding guilds and even among different species of herbivores (Bonaventure *et al.*, 2011; Heil *et al.*, 2012; Benikhlef *et al.*, 2013; Appel and Cocroft, 2014). Here, we used an elicitation procedure in which the amount of wounding was held constant and puncture wounds were treated either with water or oral secretions to identify distinct responses in photosynthesis associated with the oral secretion treatment. Specifically, while both wounding and OS-elicitation decreased A_C and g_s in local leaves, only OS-elicitation resulted in these responses in systemic leaves (Fig. 2). A recent study of photosynthesis responses in *D. wrightii* plants found that this species responded differentially to *M. sexta* larvae collected in nature compared with larvae reared in the laboratory (Barron-Garrford *et al.*, 2012). Our results failed to show differences in the elicited photosynthetic responses of *N. attenuata* between the oral secretions collected from field and colony-reared larvae. It is possible that the different treatment methods used might explain the different outcomes. In this study, we used standardized treatments, whereas Barron Garrford *et al.* (2015) allowed larvae to feed for 1 h on *Datura* leaves, and hence, variables other than differences in oral-secretion composition, such as herbivore behaviour or the effects of iterative damage, might be at play.

Previously, it was shown that *M. sexta* oral secretion, but not oral secretion from the generalist *Spodoptera littoralis* or mechanical wounding increased levels of JA in irMPK4 relative to WT plants (Hettenhausen *et al.*, 2013). NaMPK4 suppresses an essential anti-*M. sexta* JA-Ile/COI1 independent defence pathway (e.g. TPI), which affects only *M. sexta* but not *S. littoralis*. As discussed previously, TPIs appear to be regulated in parallel to A_C and g_s responses, and hence, it is likely that oral secretion-elicited photosynthesis responses may also differ among herbivores.

This specificity many reflect the ability of some herbivores to hijack plant responses in order to restrict the effectiveness of plant defences. For example, decreasing A_C (Fig. 1) likely reduces the resources for defence, and reducing g_s may regulate emissions of green leaf volatiles, as the emission of volatiles after herbivory is regulated by stomata in some species (Seidl-Adams *et al.*, 2015). Plants use volatiles as indirect defences to attract natural enemies of attacking herbivores. Reductions in the quantities of volatiles released may decrease the effectiveness of these indirect defences, but other ecological consequences of reducing g_s may also be important. Stomatal closure reduces water loss, and a rapid early response in g_s might help to economize water usage, but would also influence the deployment of direct defences, such as nicotine, which is mobilized from their sites of synthesis in the roots to damaged leaves by apoplastic transport (Baldwin 1989). All of these inferences about the ecological consequences of the observed oral secretion-elicited changes in photosynthesis require much additional work for their rigorous testing.

CONCLUSION

Responses in photosynthesis have often seen as a consequence of the damage caused by the feeding herbivore rather than an active process elicited in plants in response to perceiving the attack from herbivores. We show that *N. attenuata* plants after perceiving herbivore-associated elicitors actively modulate photosynthesis, likely well in advance of the biochemical and metabolic reconfigurations thought to induce changes in photosynthesis. We dissect the early photosynthetic responses of plants and demonstrate a specific regulation of A_C by the perception of herbivore elicitors in local and systemic leaves of

N. attenuata. These responses, which result from changes in g_s, result from OPDA signalling in local leaves, and the interplay of ABA, CK and JA/JA-Ile signalling in systemic leaves. Together with previous studies, our results suggest that herbivore-elicited photosynthesis-responses comprise at least three phases: (1) an early phase with changes in g_s, regulated by phytohormones after wounding and perception herbivore elicitors; (2) changes in Rubisco carboxylation activity that may occur in response to significant losses in photosynthetic capacity; and (3) in RuBP turnover that result from source sink and metabolic reconfigurations (Thomson *et al.*, 2003; Nabity *et al.*, 2009). From this work, it is abundantly clear that the early responses to herbivore attack are not simply a consequence of the damage by herbivores but are rather active processes elicited in plants in response to perceiving the attack by herbivores often seen as a consequence of the damage caused by the feeding herbivore rather than an active process elicited in plants in response to perceiving the attack from herbivores.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Figure S1. Intracellular CO2 levels remain unchanged after wounding and oralsecretion-elicitation. Estimated Ci rates of gas-exchange measurements at 400 ppm CO2 of local and systemic leaves of N. attenuata wild type plants 4h after wounding (WW, blue boxes) or M. sexta oralsecretion-elicitation (C, black boxes) and untreated control (WOS, white boxes). Significant differences were analyzed by one-way ANOVAs.
Figure S2. Rubisco activity and RuBP regeneration remained unaffected, while stomatal responses changed after wound and herbivore elicitation. Plants were treated by wounding (WW, blue) or M. sexta oral-secretion-elicitation (WOS, black) or not (control; C, white) and leaf gas-exchange responses at different CO2 levels were measure in treated (local) leaves and vascular connected non-treated (systemic) leaves of N. attenuata. (a) Carbon assimilation to intercellular CO2 (AC/Ci) (mean ± SEM) and (b) transpiration to stomatal conductance response curves of local and systemic leaves of N. attenuata plants are shown. Mean linearity of the ribulose-1,5-bisphosphate carboxylase/oxygenase activity phase is displayed for each treatment (C, dash line; WOS, solid; WW, blue line). Slope (k) and multiple R-square correlations (MRS) are displayed in the graph. Statistical differences of AC/Ci phases were tested by multiple MANOVA.
Figure S3. Elicited leaf gas-exchange responses are comparable between oral secretions of laboratory- and wild-M. sexta larvae. Photosynthetic responses measured in systemic leaves of untreated control N. attenuata plants (C, white) and treated by wounding (WW, blue) or M. sexta oralsecretion- elicitation (WOS, black) from M. sexta larvae collected from a colony reared for more than 200 generations in the 'laboratory' or (2) 'wild' larvae collected from native plants at the field station in Utah (details in Materials and Methods). (a) Shows carbon assimilation, transpiration and stomatal conductance to water vapor (gs) responses at 400 ppm of ambient CO2. Statistical differences were tested with two-way ANOVAs. Asterisks indicate significant differences between control and treated samples (Tukey's HSD post hoc test, ***p<0.001, **p<0.001, *p<0.05, # p<O.1). (b) Shows carbon assimilation to intercellular CO2 (AC/Ci) response curve. Lines describe mean linear response of the Rubisco activity phase for each treatment (C, dash line; WW, solid blue line; WOS-laboratory colony, solid line; WOS-wild colony, dash-dot line). Slope and correlation coefficients are displayed in the graph.
Figure S4. Accumulation of jasmonates and cytokinin metabolites induced by wounding and OS. Leaf samples of N. attenuata plants were collected 1h after treatment by wounding (WW, blue boxes) or M. sexta oral-secretion-elicitation (WOS, black boxes) and controls (C, white boxes) and plant-hormone metabolites were analyzed by LQMS. Jasmonates: hydroxyl-JA (OH-JA), glucosylated-JA (JAG), 12-hydroxyl-jasmonoyl-isoleucine (OH-JA-Ile), dicarboxy-jasmonoyl-isoleucine (COOH-JA-Ile). Cytokinins: isopentenyl adenine (IP), isopentenyl adenine riboside (IPR), isopentenyl adenine 7-glucoside (IP7G), cis-zeatin (cZ), cis-zeatin riboside (cZR), cis-zeatin riboside 7-glucoside (cZR7G), cis-zeatin 9- glucoside (cZ9G), trans-zeatin (tZ), trans-zeatin riboside (tZR), trans-zeatin riboside 7-glucoside (cZR7G), trans-zeatin 9-glucoside (tZ9G). Statistical differences were tested with two-way ANOVAs. Asterisks indicate significant differences between control and treated samples (Tukey's HSD post hoc test, ***p<0.001, **p<0.001, *p<0.05, # p<O.1).
Figure S5. Stomatal responses regulated by NaMPK4 induced by oralsecretion- elicitation and CO2 are localized in shoots, while silencing NaMPK4 roots affects water relations and sugar (ribulose 1-5 bisphosphate) turnover. Plants were treated by wounding (WW, blue) or M. sexta oralsecretion-elicitation (WOS, black) or not (control; C, white) and leaf gas-exchange responses at different CO2 levels were measured in treated (local) leaves and vascularly connected non-treated (systemic) leaves of N. attenuata. (a) Carbon assimilation to intracellular CO2 (AC/Ci) (mean ± SEM) response curves for lines silenced in N. attenuata mitogen-activated protein kinase-4 (irMPK4) and grafts of WT/irMPK4 (shoot/root). (b) Transpiration to stomatal conductance (gs) analysis of AC/Ci curves. Rubisco activity phase and E/gs relation was tested by general linear models. Statistical differences were tested by multiple MANOVAs.
Figure S6. Impaired CK perception and ABA-MPK4 signalling changed stomatal behaviour and RuBP-turnover responses to oral-secretion-elicitation in local leaves. Plants were treated by wounding (WW, blue) or M. sexta oral-secretion-elicitation

(WOS, black) or not (control; C, white) and leaf gas-exchange responses at different CO2 levels were measure in treated (local) leaves and vascular connected non-treated (systemic) leaves of N. attenuata. (a) Carbon assimilation to intracellular CO2 (AC/Ci) (mean ± SEM) response curves of N. attenuata silenced lines in CK receptors CHASE-containing Histidine Kinase 2 and 3 (irCHK2/3) and crosses of transgenic lines silenced in N. attenuata mitogen-activated protein kinase-4 (irMPK4) and irCHK2/3 lines (irMPK4*irCHK2/3). (b) Transpiration to stomatal conductance (gs) analysis of AC/Ci curves for irCHK2/3 and irMPK4*irCHK2/3 lines. Rubisco activity phase and E/gs relation was tested by general linear models. Statistical differences were tested by multiple MANOVAs.

Figure S7. 12-oxophytodenoic acid but not jasmonic acid signaling regulates transpiration to stomatal conductance and sugar (ribulose 1-5 bisphosphate turnover) responses to herbivore elicitation. Plants were treated by wounding (WW, blue) or M. sexta oral-secretion-elicitation (WOS, black) or not (control; C, white) and leaf gas-exchange responses at different CO2 levels were measured in treated (local) leaves and vascular connected non-treated (systemic) leaves of N. attenuata. (a) Carbon assimilation to intracellular CO2 (AC/Ci) (mean ± SEM) response curves for irAOC and irACX1 lines. (b) Transpiration to stomatal conductance (gs) analysis of AC/Ci curves for irAOC and irACX1 lines. Rubisco activity phase and E/gs relations were tested by general linear models. Statistical differences were tested by multiple MANOVA.

Figure S8. Intracellular CO2 rates are not affected after oral-secretion-elicitation in lines silenced in N. attenuata mitogen-activated protein kinase-4 (irMPK4) or CK receptors CHASEcontaining Histidine Kinase 2 and 3 (irCHK2/3). N. attenuata irMPK4, irCHK2/3, irMPK4*irCHK2/3 and WT/irMPK4 (shoot/root) grafted plants were treated by wounding (WW, blue) or M. sexta oral-secretionelicitation (WOS, black) or not (control; C, white) and leaf gas-exchange responses at 400 ppm of CO2 levels were measured in treated (local) leaves and vascular connected non-treated (systemic) leaves. Figure displays calculated values of transpiration and estimations of Ci levels after treatments. For comparison, the averages of the responses observed in the wild type (WT) plants (Fig. 1) are indicated by color code arrows along the y-axis. Significant differences were detected by one-way ANOVA. Asterisks indicate significant differences between control and treated samples (Tukey's HSD post hoc test, ***p<0.001, ** p<0.001, *p<0.05, # p<O.1).

Figure S9. Silencing N. attenuata mitogen-activated protein kinase-4 (NaMPK4) increases the accumulation levels of 12-oxo phytodenoic acid (OPDA), jasmonic acid (JA) and JA-isoleucine (JA-Ile). Leaves of WT and irMPK4 plants, treated with wounding (WW, blue) or M. sexta oral-secretionelicitation (WOS, black) and control (C, white), were collected 1h after treatment and hormone accumulation was determined by LQMS. Differences were tested with two-way ANOVA (treatment*line/tissue) for every phytohormone. Asterisks indicate significant differences between control and treated samples (Tukey's HSD post hoc test, ***p<0.001, ** p<0.001, *p<0.05, # p<O.1). JA eq, jasmonic acid equivalents; FM, fresh mass.

Figure S10. Photosynthetically active radiation (PAR) on Utah field station. PAR was measured using a Specbos 1211UV (JETI Technische, Instrumente GmbH) on June 2nd, 2013 in the Utah field station (37°16'10.49" N/113°16'53.15" W, Utah, USA).

Figure S11. Putative aquaporin gene expression is regulated by wounding and oral-secretion-elicitation in leaves and roots of N. attenuata. Gene expression was measured in leaves at several time points (0.5, 1, 5, 9,13, 17 and 21h) after wounding (WW, dashed line) or M. sexta oralsecretion- elicitation (WOS, solid line) and in untreated control leaves (C, dotted line). Data was extracted from the kinetic microarray-analysis (Kim et al., 2011). Error bars show standard errors (n=3). Accession numbers (code below gene name) can be found in GEO-NCBI (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE30287).

