J Plant Growth Regul (2010) 29:289–296
DOI 10.1007/s00344-009-9135-2

Cytokinin Primes Plant Responses to Wounding and Reduces Insect Performance

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Received: 14 August 2009 / Accepted: 9 December 2009 / Published online: 20 January 2010

Abstract We report a potential role of endogenous cytokinin supply in priming plant defense against herbivory. Cytokinin priming significantly reduced weight gain by insect larvae. Unlike previously described priming by volatile compounds, priming by cytokinin did not overcome vascular restrictions on systemic wound signaling. However, similar to priming by volatile compounds, cytokinin priming occurred upstream of accumulation of jasmonic acid and its precursor linolenic acid in mechanically wounded source leaves. Cytokinin priming significantly altered wound-induced accumulation of transcripts encoding homologs of allene oxide synthase, trypsin inhibitor, and chitinase. Cytokinin priming may reflect coordination between aboveground inducible defense against herbivory and belowground processes such as nutrient availability. These findings should encourage investigations of how genetic alterations in cytokinin signaling and response pathways may affect plant–herbivore interactions.

Electronic supplementary material The online version of this article (doi:10.1007/s00344-009-9135-2) contains supplementary material, which is available to authorized users.

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Keywords Cytokinin · Priming · Wounding · *Populus*

Introduction

Priming is a physiological process by which a plant is conditioned for a more rapid or higher-magnitude response to a subsequent event (Bostock 2005; Conrath and others 2006; Frost and others 2008a). With regard to responses against herbivores, the majority of previous work on defense priming has focused on herbivore-induced volatile compounds as priming signals (Engelberth and others 2004; Frost and others 2007; Heil and Silva Bueno 2007; Ton and others 2007), largely in an effort to explain the phenomenon of plant-to-plant communication (Heil and Ton 2008; Frost and others 2008a). However, priming of systemic regions within a plant involves signals within the vasculature as well (Orians 2005; Erb and others 2008; Koornneef and Pieterse 2008). For example, nonadjacent leaves that share direct vascular connection with a wounded leaf show more rapid and/or effective defense responses after damage, relative to leaves without such a vascular connection in *Populus* (Davis and others 1991; Jones and others 1993; Orians and others 2000; Schittko and Baldwin 2003; Gomez and Stuefer 2006; Frost and others 2007). In addition, prior foliar herbivory and prior root herbivory incite spatially distinct patterns of antiherbivore defenses within the foliage of *Gossypium* (Bezemer and others 2003, 2004), implying there may be diverse mechanisms underlying vascular priming. Moreover, the physiological and molecular mechanisms that enable priming are not clear.

There is evidence that cytokinin can play a role in integrating diverse environmental stress responses (Hare and others 1997); however, plant responses to wounding

and herbivory are generally coordinated by the phytohormones jasmonic acid (JA), abscisic acid, auxin, and ethylene (Kessler and Baldwin 2002; Conrath and others 2006; Koornneef and Pieterse 2008; Erb and others 2008, 2009; Wu and Baldwin 2009). Despite these general trends, cytokinin has been implicated as having a role in wound-inducible gene expression. Transgene-mediated cytokinin overproduction led to elevated levels of transcripts encoding chitinase, extensin, and PR-1, but these effects were ascribed to stress responses given the altered morphology of the plants (Memelink and others 1987). Mechanical wounding increased cytokinin concentration and endogenous cytokinin activity in potato tubers and cucumber cotyledons, which was proposed to reflect a potential role for cytokinin in stimulating cell division and tissue repair (Mitchell and van Staden 1983; Crane and Ross 1986). Herbivory by *Manduca sexta* may stimulate cytokinin signaling in wild tobacco based on elevated abundance of transcripts for *cig2* (a cytokinin-induced gene; Hui and others 2003).

Some observations suggest a role of cytokinin in priming of antiherbivore defenses. Conditional cytokinin biosynthesis directed by a wound-inducible transgenic construct was sufficient to deter insect feeding and delay larval development (Smigocki and others 1993) and caused higher inducibility of an endogenous cytochrome P450 gene (Mujer and Smigocki 2001). In addition, transgenic tobacco plants with elevated endogenous cytokinin levels, a consequence of overexpression of a gene (*rgp1*) encoding a small GTP-binding protein, showed increased JA accumulation relative to wild-type plants, and the short-term exposure of wild-type tobacco plants to exogenous cytokinin accelerated the timing of wound-induced JA accumulation (Sano and Ohashi 1995; Sano and others 1996).

Our present study was performed to evaluate the impact of cytokinin in priming local and systemic wound-responsive gene expression and subsequent effects on a generalist insect herbivore. The experiments were conducted with *Populus*, which is a useful experimental system for this work (Bradshaw and others 2000; Jansson and Douglas 2007), particularly because assimilate movement within the vascular system of developing leaves has been well-defined (Larson and Dickson 1973; Isebrands and others 1976; Vogelmann and others 1982). Along with assimilates, sink leaves import wound-associated signals from source leaves, which is one means by which herbivory-associated cues perceived in source leaves are transduced to developing leaves (Davis and others 1991; Jones and others 1993). Indeed, sink status per se may be a key factor in the inducibility of systemic wound responses (Arnold and Schultz 2002; Arnold and others 2004). Movement of wound signal generally flows from source to sink due to vascular constraints (Orians 2005), but it can also occur bidirectionally among leaves that share direct vascular connections (Jones and others 1993).

We report that cytokinin primes plant responses to wounding, and such priming reduces insect larval weight gain in systemically responsive leaves. The priming effect of cytokinin is not associated with a disruption in the normal pattern of carbon assimilate movement within the developing leaf zone but appears to occur upstream of JA biosynthesis and expression of wound- and herbivory-induced genes. Because cytokinin is a phytohormone originating in roots and transmitted to shoots, cytokinin priming may reflect a means by which aboveground antiherbivore defense responses can be coordinated with belowground signals.

Materials and Methods

Plant Material and Treatments

Poplars were propagated as rooted shoot apex-derived cuttings under mist, grown in 3.87-l pots, and used in experiments after achieving a height of 60–80 cm. Insect feeding assays were performed on the F_1 hybrid poplar genotype H11-11 (*Populus trichocarpa* Torr. and Gray × *P. deltoides* Bartr. ex Marsh.) grown in chambers maintained at 23°C under 16 h light/8 h dark cycles as described previously (Lawrence and others 1997). Assimilate movement, phytohormone, and transcript abundance experiments were performed on hybrid H11-11 or the sequenced reference genotype Nisqually-1 (*Populus trichocarpa* Torr. and Gray; Tuskan and others 2006) trees grown under greenhouse conditions in Gainesville, FL, with supplemental lighting and fertigation provided as described previously (Ramírez-Carvajal and others 2009) to maintain continuous growth.

Leaves were identified by leaf plastochron index (LPI; Larson and Isebrands 1971). The index leaf (LPI 0) is near the shoot apex and has a blade that is half unfolded and approximately 3.5 cm in length. Leaves are numbered consecutively and positively down the stem. When used in experiments, plants had between 20 and 25 leaves. 6-Benzylaminopurine (BAP) was dissolved in 2 ml of 0.1 M NaOH then diluted to 1 L for a final concentration of 100 μM. BAP or 200 μM NaOH (control) was applied to leaves as a spray until runoff, a total of about 25 ml per plant. This equates to about 100 nmol BAP applied per leaf, about 20 nmol BAP applied per gram fresh weight (FW) of leaf tissue, and approximately 2.5 μmol BAP applied per plant. The endogenous concentration of aromatic cytokinins in *Populus* foliage was estimated to be 80 pmol g^{-1} FW (Novak and others 2008), which is equivalent to about 0.5% uptake of applied BAP into leaves in our experiments. The effects of cytokinin priming

(cytokinin vs. control) were assessed in experiments that commenced after these treatments. Leaves were wounded with pliers. Each plier "bite" crushed an approximately 1-cm^2 area of leaf, and 20–30 wounds were distributed randomly throughout the lamina (avoiding the midvein) such that about one-third of the lamina was damaged. Experiments were implemented as completely randomized designs, and analysis of variance was implemented in SAS (SAS Institute, Cary, NC) to assess treatment effects.

Phytohormones and Transcript Abundance

Plants were treated with cytokinin or mock solution as described above. After 24 h, source leaves at LPI 6–11 were wounded, and source leaves at LPI 12–14 were collected ("0 h"). At 6 h after wounding, leaves at LPI 6, 8, and 10 were harvested ("6 h"). At 24 h after wounding, leaves at LPI 7, 9, and 11 ("24 h") along with sink leaves at LPI 1–5 ("systemic") were harvested. All samples were flash frozen in liquid nitrogen immediately after excision and then stored at −80°C until extractions were performed.

Extraction and quantification of JA and linolenic acid (LNA) were performed as previously described (Frost and others 2008b). Briefly, approximately 100 mg frozen leaf tissue of each sample (Nisqually-1) was ground with mortar and pestle and transferred to FastPrep tubes (Qbiogene, Carlsbad, CA) containing Zirmil beads (SEPR Ceramic Beads and Powders, Mountainside, NJ). Dihydro-JA (CDN Isotopes, Pointe-Claire, Quebec, Canada) and gamma-LNA (Ultra Scientific, North Kingstown, RI) were added as internal standards (100 ng each). The samples were mixed with 1-propanol:H_2O:HCl (2:1:0.002) and homogenized in a FastPrep FP 120 (Qbiogene). Dichloromethane was quickly added to each sample, which were shaken and centrifuged. The bottom organic phase (dichloromethane) was transferred to a glass screw-cap vial and evaporated by a constant airstream. Each sample was reconstituted in diethyl ether:methanol (9:1 v/v), and carboxylic acids were converted into methyl esters using a trimethylsilyldiazomethane (Sigma–Aldrich, St. Louis, MO). Volatile metabolites were separated from the complex mixture by vapor-phase extraction and analyzed by chemical ionization–GCMS as previously described (Schmelz and others 2003, 2004).

Transcript abundance was quantified using real-time quantitative reverse transcription PCR as described previously (Ramírez-Carvajal and others 2008). Briefly, total RNA was isolated, treated with DNase, and reverse transcribed using an oligo(dT) primer. Transcript abundance was quantified in three aliquots of the same cDNA reaction (technical replications) from each of four different plants per treatment (biological replications) using the SYBR® Green kit (Stratagene, La Jolla, CA) in the Mx3000P thermocycler (Stratagene). Gene-specific primers (Supplementary Table 1) were identified using the Joint Genome Institute assembly for *P. trichocarpa* with NetPrimer (Premier Biosoft International, Palo Alto, CA), and melt curve analysis was used to verify single products. Protein abundance in hybrid H11-11 was analyzed by Western blotting (Supplementary Fig. 1).

Insect Performance

Gypsy moth eggs (*Lymantria dispar*) were obtained from USDA-APHIS (Otis International Guard Base, MA) and maintained at 25°C in the dark on artificial diet. When larvae reached the second instar they were placed in empty diet cups to starve for 24 h. Lower leaves (LPI 8 and older) were mechanically wounded at 21 and 24 h after cytokinin treatment, after which ten second instar larvae were placed on each leaf at LPI 3–6 and covered with a fabric bag. Larvae fed for 4 days after which they were collected and weighed.

Assimilate Movement

Plants were treated with cytokinin or mock solution as described above. At 24 h after treatment, carbon radiolabeling experiments were performed on hybrid H11-11 as described previously (Davis and others 1991). Briefly, the leaf at LPI 8 was wounded and a total of 0.44 MBq of ^{14}C-labeled sucrose (3.7 MBq/ml; NEC100X, PerkinElmer, Shelton, CT) in 0.01% Regulaid (Kalo, Inc., Overland Park, KS) was then applied in 5-µl droplets to the underside of the leaf. After 6 h, LPI 8 and younger leaves were harvested and ^{14}C was measured in extracts.

Results

Cytokinin Primes JA Biosynthesis

Diverse priming agents, including volatile compounds, are known to prime JA biosynthesis (Engelberth and others 2004; Conrath and others 2006; Frost and others 2008a). Consequently, we measured JA and its fatty acid precursor linolenic acid (LNA) in wounded leaves (Fig. 1) and found that cytokinin increased the wound-inducible accumulation of JA and LNA to an extent similar to priming by the green leaf volatile *cis*-3-hexenyl acetate (Frost and others 2008b). Consistent with a role in priming, cytokinin treatment alone did not alter baseline levels of JA or LNA in unwounded leaves (Fig. 1, 0 h). These data imply the mechanism of cytokinin priming occurs upstream of JA biosynthesis and upstream of LNA accumulation in wounded leaves.

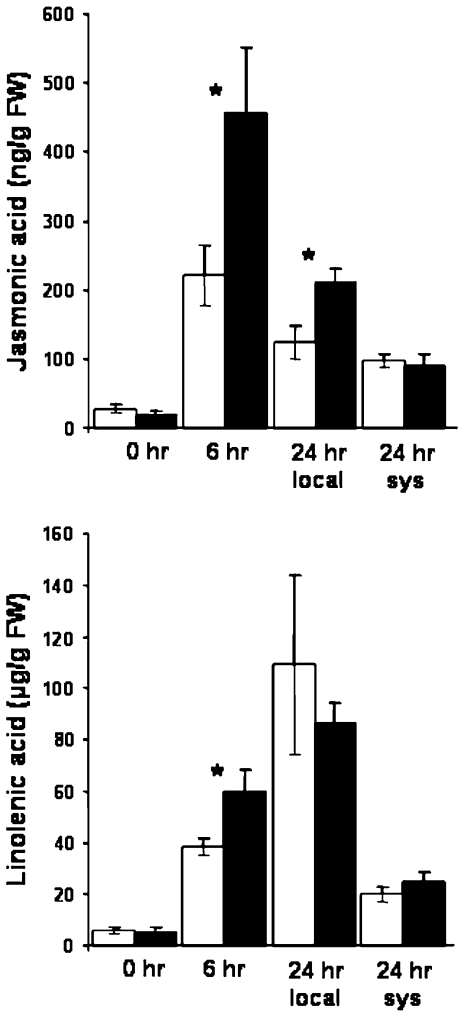

Fig. 1 Cytokinin primes wound-induced jasmonic acid (JA, *upper*) and linolenic acid (LNA, *lower*) accumulation. At 24 h after mock or cytokinin treatment, leaves were wounded and samples were collected at the indicated times (four plants per treatment). Means for the mock (*open bar*) versus cytokinin (*solid bar*) contrasts were either not significant or were declared significant (* $P < 0.05$, *t* test). *Error bars* denote standard deviation

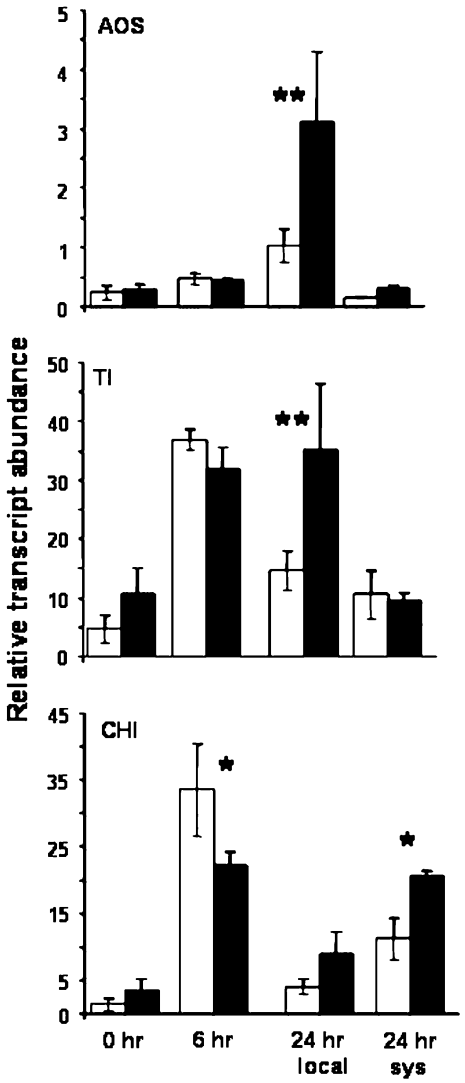

Fig. 2 Cytokinin primes wound-induced expression of allene oxide synthase (AOS), trypsin inhibitor (TI), and chitinase (CHI). At 24 h after mock or cytokinin treatment, leaves were wounded and samples were collected at the indicated times. Real-time quantitative PCR was performed on three aliquots of each first-strand cDNA sample (technical replication) and on four plants per treatment (biological replication). Transcript abundance of each gene is expressed relative to *ACTIN2* abundance. Means for the mock (*open bar*) versus cytokinin (*solid bar*) contrasts were either not significant or were declared significant (* $P < 0.05$; ** $P < 0.01$, *t* test). *Error bars* denote standard deviation

Cytokinin Primes Accumulation of Wound-induced Transcripts

We then investigated the abundance of transcripts encoding allene oxide synthase (AOS), *win3* trypsin inhibitor (TI), and *win8* chitinase (CHI) using real-time quantitative RT–PCR. For AOS and TI, transcript accumulation either did not differ (0, 6, and 24 h systemic) or was significantly higher (24 h local) in cytokinin-treated plants compared to mock-treated plants (Fig. 2; $P < 0.01$). Cytokinin stimulated systemic CHI transcript accumulation by 85% ($P < 0.05$). The overall magnitude of the cytokinin priming effect was typically twofold, which is smaller in magnitude than the wound response observed with these transcripts (Fig. 2) (Lawrence and others 2006). The priming effect is also apparent in accumulation of wound-induced proteins (Supplementary Fig. 1).

Cytokinin Priming Reduces Caterpillar Weight Gain

To address the potential ecological significance of cytokinin priming, we measured gypsy moth larval growth after cytokinin treatment. Wounding and cytokinin significantly affected the weight of gypsy moth larvae, but the

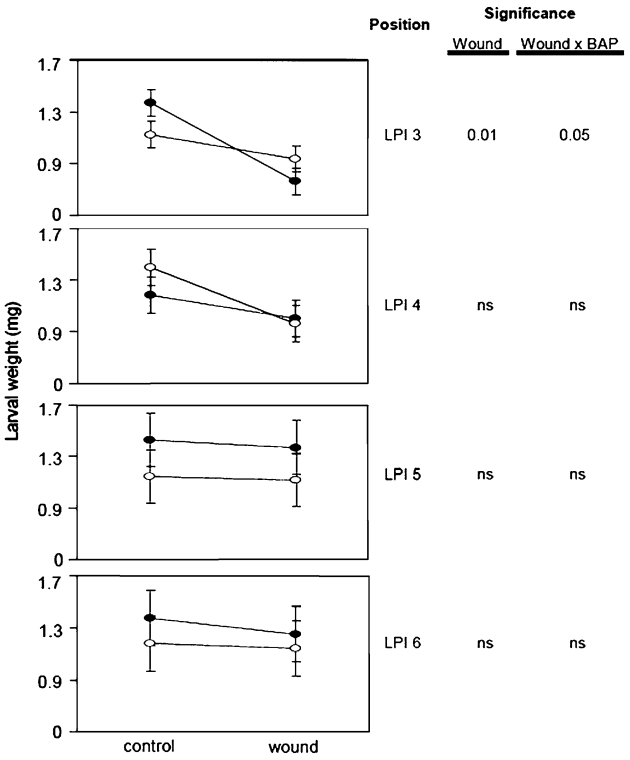

Fig. 3 Wounding and cytokinin treatment reduce weight of second instar gypsy moth larvae. Open circles represent water-treated plants and shaded circles represent cytokinin-treated plants. Leaf position within the developing leaf zone is indicated from sink (LPI 3) to source (LPI 6). The experiment was conducted as a 2 × 2 factorial arrangement of treatments (wounded vs. unwounded; mock vs. cytokinin) in a completely randomized design with three plants (biological replicates) per treatment. The effects of cytokinin and leaf position on larval weight gain were not statistically significant. *Error bars* denote standard deviation and asterisks denote significant differences (*F* test)

manifestation of the cytokinin priming effect was dependent on the position of the feeding leaf within the sink-to-source gradient from LPI 3 (sink) to LPI 6 (source; Fig. 3). The main effect of wounding was highly significant ($P < 0.01$) in larvae that consumed LPI 3 leaves but not larvae that consumed LPI 5 or 6 leaves. The biological interpretation of the significant wound × cytokinin interaction in LPI 3 leaves ($P < 0.05$) is that cytokinin priming exerted a deleterious effect on larvae that consumed LPI 3 leaves. The results imply that the drivers of this interaction could be cytokinin-induced susceptibility in control LPI 3 leaves, cytokinin priming in wounded LPI 3 leaves, or some combination of both factors. This interaction effect was not significant for larvae feeding on leaves at LPI 4, 5, or 6.

Larvae feeding on unwounded plants, averaged across cytokinin treatment, weighed the same regardless of the leaf on which they fed (range = 1.25-1.29 mg, mean differences n.s.), indicating no effects of leaf ontogeny on larval weight in unwounded leaves in this study. Similarly, cytokinin treatment exerted no significant impact on larval weight regardless of leaf position; the largest mean difference was for LPI 5 (1.13 mg in mock-treated plants and 1.40 mg in cytokinin-treated plants), but these differences were not statistically significant.

Cytokinin Treatment Does Not Enhance Sink Strength of Importing Leaves

We reasoned that because cytokinin can promote the sink strength of developing organs (Roitsch and Ehneβ 2000), cytokinin might increase source-to-sink assimilate movement, or perhaps alter vascular constraints on assimilate movement. Sink leaves therefore might import more assimilates as well as wound signal from wounded source leaves. Because cytokinin-induced variation in sink strength could drive the effects we observed, we measured assimilate import from LPI 8 into the developing leaf zone at 6 h, the time point at which (based on the priming of JA) the plants were demonstrating a priming effect. If sink strength explained the priming effect, we reasoned that cytokinin treatment would increase assimilate import into sink leaves at the time during which priming was evident. We found that cytokinin did not alter patterns of assimilate import (Fig. 4), nor did it stimulate an increase in absolute quantities of imported assimilate compared to mock controls (data not shown). To the contrary, there was weak

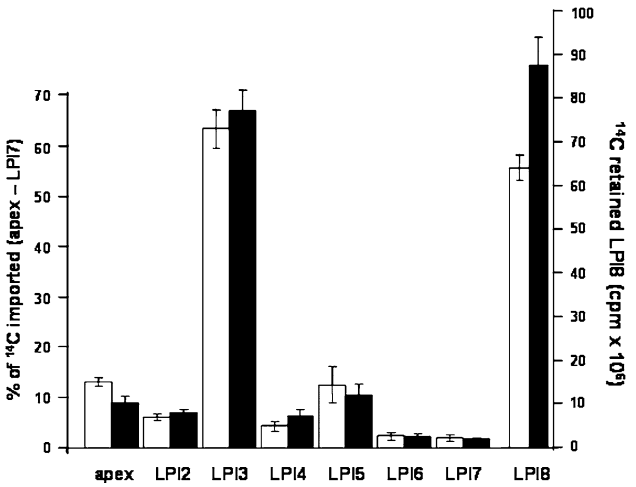

Fig. 4 Cytokinin does not significantly increase sink strength of developing leaves, nor does it overcome vascular constraints. At 24 h after mock or cytokinin treatment, radiolabeled sucrose was applied to the underside of leaves at LPI 8. After 6 h, individual leaves shown and the shoot apex (LPI 1 and above) were harvested for quantification of radiolabel. Means for the mock (*open bar*) versus cytokinin (*solid bar*) contrasts were not significantly different (four plants per treatment). The experiment was conducted with a 2 × 2 factorial arrangement of treatments (wounded versus unwounded; mock versus cytokinin) as a completely randomized design. There was weak evidence for cytokinin promoting retention of ^{14}C by LPI 8 ($P = 0.08$, *t* test). *Error bars* denote standard deviation

evidence that cytokinin-treated LPI 8 leaves may retain more assimilate than mock controls ($P = 0.08$). Wounding exerted no influence on assimilate movement (data not shown). These results implied that cytokinin stimulation of source-to-sink assimilate movement is unlikely to explain cytokinin priming.

Discussion

Inducible defenses are one form of phenotypic plasticity, a property thought to reflect an adaptation to patchy, variable, and unpredictable environmental conditions (Scheiner 1993; Agrawal 2001; Orians and Jones 2001; Fordyce 2006). The capacity of a host plant to detect and rapidly respond to herbivory, which can be quite variable in space and time, is therefore considered adaptive. In such variable environments, it appears that there is also a benefit to being able to prepare for future potential events. Because this is a dynamic event, priming falls within the category of an inducible response (Frost and others 2008a). Primed tissues can mount an accelerated and more vigorous response in case of actual herbivory. Previous work has identified airborne signals that may serve as priming agents. In poplar trees, priming of herbivory-responsive genes by the herbivore-induced plant volatile compound *cis*-3-hexenyl acetate (Frost and others 2008a, b) appears to accelerate and expand defensive capacity of undamaged neighboring organs. Other plants also apparently can use volatile compounds as signals that initiate priming responses (Heil and Silva Bueno 2007). Our results suggest cytokinin may warrant further consideration as a regulator of priming within the vasculature.

In this article we present evidence that cytokinin can prime plant responses to wounding and herbivory. Wound-responsive JA and LNA and transcripts for AOS, TI, and CHI were primed by cytokinin, but not assimilate movement from wounded source leaves to unwounded sink leaves. This implies cytokinin priming may influence wound signal generation or perception more than wound signal movement from source to sink leaves. Of course, unlike priming by volatile molecules, cytokinin is unlikely to overcome vascular constraints within an individual plant or provide a wound signaling cue between plants. Rather, our results suggest that cytokinin is involved in defense priming within a plant. The potential ecological relevance of cytokinin priming is supported by the significance of the wound × cytokinin interaction—beyond the significant effect of wounding alone—on larval weight gain. It should be noted that the wound × cytokinin interaction is driven by differential responses of unwounded versus wounded plants to cytokinin. If cytokinin were to repress defense responses in unwounded leaves, then in this respect the cytokinin priming mechanism may differ from that of other priming agents.

Our results also suggest that cytokinin may prime octadecanoid biosynthesis by different mechanisms than do volatile compounds in poplar because AOS transcripts were not primed by *cis*-3-hexenyl acetate (Frost and others 2008b). Moreover, the roles of cytokinin and JA are clearly distinct because methyl-JA application was sufficient to induce accumulation of poplar transcripts encoding TI (Haruta and others 2001) and vegetative storage protein (Davis and others 1993) in the absence of wounding or herbivory, whereas cytokinin primes but does not induce gene expression in the absence of wounding. Interestingly, the cytokinin-induced priming response can be detected in JA accumulation (6 h) before it is detectable in AOS transcript accumulation (24 h), suggesting that either the usual AOS induction is capable of generating increased amounts of JA or that the JA is being synthesized via another route, such as the direct release of 12-oxo-10,15(Z)-octadecatrienoic acid (OPDA) from membranes (Stelmach and others 2001).

Cytokinin priming of wound responses may reflect one way that physiological and metabolic cues could impinge on antiherbivore defense in foliage, though the mechanisms by which this occurs are not understood (Bezemer and van Dam 2005; Erb and others 2008; van der Putten and others 2009). Cytokinin signaling is linked to nutrient availability and nitrogen status, which can have significant effects on host development and herbivory. Thus, it is reasonable to hypothesize that cytokinin-mediated priming may be dependent on—or at least reflective of—the nutrient status of a plant. However, support for such a hypothesis is equivocal and in need of further attention. Increased nitrogen availability resulted in higher JA biosynthesis as well as downstream inducible antiherbivore genes in *Nicotiana attenuata* (Lou and Baldwin 2004), whereas higher nitrogen availability was associated with reduced JA accumulation and elevated terpenoid aldehyde accumulation in *Gossypium hirsutum* (Chen and others 2008). The reduction in JA inducibility in cotton was somewhat unexpected because JA application was sufficient to induce terpenoid aldehydes in the absence of herbivory (Opitz and others 2008). Cytokinin is proposed to be a systemic signal reflecting nutrient status based on positive correlations between cytokinin levels and nitrogen levels (Takei and others 2001, 2002; Sakakibara and Takei 2002; Sakakibara 2006). Our study provides the foundation for further investigation of the role of cytokinin in plant defense priming. An understanding of the actual mechanisms involved in priming per se, as opposed to the consequences of priming, will be required to reconcile findings from various plants and experimental systems. Furthermore, the recent development of transgenic and knockout lines for

cytokinin response pathways (Kakimoto 2003; Ferreira and Kieber 2005; Sakakibara 2006; Argyros and others 2008) should enable dissection of cytokinin priming mechanisms and evaluation of its generality among plant taxa.

Acknowledgments This research was supported by grants from the Department of Energy, Office of Science, Office of Biological and Environmental Research (DE-AC05-00OR22725 to JMD), and the United States Department of Agriculture (USDA-NRI 2007-35302-18087 to CJF).

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