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Adaptive significance of gall formation for a gall-inducing aphids on Japanese elm trees

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ABSTRACT

Insect galls are abnormal plant tissues induced by external stimuli from parasitizing insects. It has been suggested that the stimuli include phytohormones such as auxin and cytokinins produced by the insects. In our study on the role of hormones in gall induction by the aphid *Tetraneura nigriabdominalis*, it was found that feedback regulation related to auxin and cytokinin activity is absent in gall tissues, even though the aphids contain higher concentrations of those phytohormones than do plant tissues. Moreover, jasmonic acid signaling appears to be compromised in gall tissue, and consequently, the production of volatile organic compounds, which are a typical defense response of host plants to herbivory, is diminished. These findings suggest that these traits of the gall tissue benefit aphids, because the gall tissue is highly sensitive to auxin and cytokinin, which induce and maintain it. The induced defenses against aphid feeding are also compromised. The abnormal responsiveness to phytohormones is regarded as a new type of extended phenotype of gall-inducing insects.

1. Introduction

Insect-induced galls are fertile ground for the study of adaptive traits in both plants and insects. Three hypotheses have been proposed for the adaptive significance of gall induction: a nutrition hypothesis, a microenvironment hypothesis, and an enemy hypothesis (Stone and Schonrogge, 2003). The nutrition hypothesis is based on the idea that feeding on galls provides the inducers enhanced nutrition over other feeding modes. This hypothesis is consistent with gall-inducer preferences for internal structures with complex folds, which provide a greater surface area for feeding. According to the microenvironment hypothesis, gall tissues protect gall-inducing insects from unfavorable abiotic conditions, particularly desiccation (owing to a gall's closed state, outer structures that reduce its surface area, and its low permeability). The enemy hypothesis purports that galls protect gall inducers from attack by natural enemies. Although no galls keep their inducers completely safe from enemies, this hypothesis best explains the evolution and maintenance of diversity in external gall structures (Stone and Schonrogge, 2003).

Most discussions on the adaptive significance of galls have focused on gall morphological phenotype. However, gall inducers also effect changes in their host's physiology, particularly suppressing host defense responses, for their own benefit. For example, the interior tissues on which an insect feeds have been found to contain lower levels of phenolic compounds than normal tissues (thus enhancing their food value), whereas exterior gall tissues contain higher levels of such compounds (thus increasing the gall's protective value) (Nyman and Julkunen-Tiitto, 2000; Allison and Chultz, 2004). Tooker and De Moraes (2007) and Tooker et al. (2008) reported that gall-inducing insects suppress host plant production of volatiles, compounds that attract natural enemies of feeding herbivores or directly deter them. Specifically, they showed that the Hessian fly (*Mayetiola destructor*) and tephritid fly (*Eurosta solidaginis*) avoided and altered the indirect defense mechanisms of their respective host plants, which can be considered a new type of adaptive manipulation of host plants by gall-inducing insects.

Our recent studies indicate that the phytohormones auxin and the cytokinins play important roles in gall induction and maintenance for gall-inducing insects, such as the sawfly (*Pontania* sp.) and gall midge (*Rhopalomyia yomogicola*) (Yamaguchi et al., 2012; Tanaka et al., 2013). The involvement of phytohormones in gall formation has long been suggested, based on the fact that they

Abbreviations: IAA, indole-3-acetic acid; *tZ*, *trans*-zeatin; *tZR*, *trans*-zeatin riboside; iP, isopentenyl adenine; iPR, isopentenyl adenosine; JA, jasmonic acid; JA-Ile, jasmonoyl-L-isoleucine; SA, salicylic acid; LC–MS/MS, liquid chromatography/tandem mass spectrometry; GC/MS, gas chromatography/mass spectrometry.

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regulate cell division, enlargement, and differentiation (all of which occur during gall induction), and that parasitic insects contain the phytohormones, sometimes at much higher levels than in their parasitized plant tissues (Mapes and Davies, 2001a,b; Tooker and De Moraes, 2011; Straka et al., 2010; Dorchin et al., 2009; Yamaguchi et al., 2012; Tanaka et al., 2013). We recently demonstrated *de novo* synthesis of indole-3-acetic acid (IAA), an active form of auxin, from tryptophan in the sawfly and gall midge (Yamaguchi et al., 2012; Tanaka et al., 2013), and also presented evidence strongly suggesting *in vivo* synthesis of cytokinins in the sawfly (Yamaguchi et al., 2012).

Aphids are completely different from sawflies and gall midges in terms of their taxonomic affinities and, more importantly, their feeding behavior. Aphids feed on phloem sap and thus do not need to induce the growth of nutritive tissue on the internal walls of a gall; instead, they require internal vascularization of the gall. Whether, despite these differences, auxin and cytokinin are involved in aphid-induced gall formation is unknown.

We studied the aphid *Tetraneura nigriabdominalis* Sasaki belonging to the tribe Eriosomatini, which induces pouched galls on the leaves of the Japanese elm tree (*Ulmus davidiana* var. japonica). We first analyzed endogenous levels of IAA and cytokinins in the aphid and plant tissues to examine if those phytohormones are involved in gall induction by the aphid species. Thereafter, transcript levels of *AUX/IAA* and *type-A RR* genes in gall tissue were compared to levels in leaf tissue. *AUX/IAA* and *type-A RR* genes are involved in feedback regulation of auxin and cytokinin signaling, respectively, and thus these genes are sometimes used as marker genes for the phytohormone signaling (Abel and Theologis, 1996; Kakimoto, 2003). We have previously suggested that the hormonal activities are enhanced in sawfly gall tissues compared with those in control leaf tissues (Yamaguchi et al., 2012) based on an expression analysis of these genes in the host plant. In the course of these experiments, we obtained unexpected results, which suggested that aphid-induced gall tissue may be more sensitive to the phytohormones, and that this increased sensitivity may be induced by aphids for their own benefit. These possibilities prompted us to investigate the defense response of gall tissue as compared with that of control leaf tissue, and to examine whether gall tissue is less protected from insect feeding, which could be regarded as a novel 'extended phenotype' of gall-inducing insects. A typical defense response induced by herbivory is the production of volatile organic compounds (VOCs); VOCs can cause either directly deleterious effects on the herbivore, or indirect effects of the herbivore consisting of attracting its natural enemies. We compared the VOC emission from gall and control leaf tissues. Because jasmonic acid (JA) and salicylic acid (SA) have been identified as key players in VOC production by plants responding to herbivory by insects (van Poecke and Dicke, 2004; Smith and Boyko, 2007), inducibility of VOC production by JA ad SA were also examined.

2. Materials and methods

2.1. Chemicals

Stable isotope-labeled compounds [$^2H_5$]*trans*-zeatin([$^2H_5$]*tZ*), [$^2H_5$]*trans*-zeatin riboside ([$^2H_5$]*tZR*), [$^2H_6$]isopentenyl adenine ([$^2H_6$]iP), and [$^2H_6$]isopentenyl adenosine ([$^2H_6$]iPR) were purchased from OlChemIm Ltd. (Olomouc, Czech Republic), [$^{13}C_6$]IAA was purchased from Cambridge Isotope Laboratories, Inc. (Andover, MA), and [$^2H_6$]methyl jasmonate (2,4,4-d_3, acetyl-d_2) was purchased from Kanto Chemical Co., INC (Tokyo, Japan). [$^{13}C_{11}$,$^{15}N_2$]L-tryptophan was donated by Dr. Kasahara (RIKEN Plant Science Center), who had purchased it from Sigma–Aldrich. Stable isotope-labeled JA was prepared by hydrolyzing [$^2H_6$]methyl jasmonate by incubation in 2:1 mixture of methanol and 3 M aqueous sodium hydroxide for 20 min at ambient temperature. During this reaction, ^2H was partly exchanged with ^1H by keto-enol tautomerism, and [$^2H_2$] and [$^2H_3$]JA were the major products; non-labeled JA ([$^2H_0$]JA) was not detected. ^2H-labeled jasmonoyl-L-isoleucine was synthesized from the ^2H-labeled JA according to the method in Jikumaru et al. (2004).

2.2. Materials for analyses of endogenous phytohormones, real-time RT-PCR, and VOCs

Galls induced by the aphid (*T. nigriabdominalis*) and leaves were collected from Japanese elm tree (*U. davidiana* var. japonica) growing in the field of Ibaraki University. This gall is called 'harunire-ha-fukuro-fushi' in Japanese according to the nomenclature convention that combines the host plant name, the galling site on the host plant, the appearance of the gall (its shape and/or color), and the word for 'gall', *i.e.*, 'harunire' means Japanese elm tree, 'ha' means leaf, 'fukuro' means pouch, and 'fushi' means insect gall. Fig. 1A shows the appearance of this gall. Aphid fundatrices start feeding on phloem sap from the abaxial side of newly formed young leaves in mid April (Fig. 1B and C). The feeding site on the leaf soon starts to cave to the opposite side (adaxial side) of the aphid, eventually enclosing the aphid in a hollow (Fig. 1D). The size of the gall increases and the entrance to the gall closes. The fundatrices continue to molt in the gall (Fig. 1E) and the 4th instar produces the winged second generation (Fig. 1F). At this stage, gall development had already ceased, suggesting that the second generation is not involved in gall induction. The second-generation aphids escape from the gall and move to the second host, the roots of Gramineae plants. In autumn, the sexupara emerge and deposit eggs on the trunks of the Japanese elm tree. In April, the hatched fundatrices move to the young leaves and begin gall induction (Yukawa and Masuda, 1996).

The harvested galls were dissected to separate aphids and plant tissue. Leaves without galls were used as control tissue. For some experiments, galls were divided into early and late stages of growth; the early-stage galls contained 1st and 2nd instar fundatrices and the late stage galls, 3rd and 4th instar fundatrices. The early and late galls were obtained at 1–2 weeks and 2–3 weeks after gall initiation, respectively. Plant materials used for analysing defense responses (analyses of VOCs, *LOX* expression, and endogenous jasmonates) were late stage galls. Special care was taken to complete the sampling procedure within 3 min to avoid the production of wound-induced jasmonates. Phytohormone analyses were undertaken on 10–80 mg of leaf or gall tissue and 1–2 mg of insect tissue. About 50 mg of leaf or gall tissue was used for RNA extraction. For phytohormone treatment, leaf or gall tissues were spray-treated with 10 μM IAA, 10 μM iPR, or 1 mM JA. The concentrations of IAA and iPR used for the spray treatment were as reported in many previous studies (citations omitted), and promoted various phenomena regulated by phytohormones. The JA concentration used to induce volatile emissions was chosen based on previous studies, including that of Radhika et al. (2012) and citations therein. For wound treatment, a branch possessing leaves with or without galls was detached from the tree with care not to wound the tissues for analysis, and the leaf or gall tissue was wound-treated by pinking a needle at about 30 points per 2 cm^2 of the tissue. The treated tissues were incubated (see figures for time periods), harvested, weighed, immediately frozen in liquid nitrogen and stored at −80 °C.

2.3. Histological observations

Thin sections (10-μm thick) of gall tissue attached to a leaf were prepared and observed under a microscope (BZ-8100, Keyence,