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The Localization of Phytohormones within the Gall-inducing Insect *Eurosta solidaginis* (Diptera: Tephritidae)

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Abstract

The phytohormone production hypothesis suggests that organisms, including insects, induce galls by producing and secreting plant growth hormones. Auxins and cytokinins are classes of phytohormones that induce cell growth and cell division, which could contribute to the plant tissue proliferation which constitutes the covering gall. Bacteria, symbiotic with insects, may also play a part in gall induction by insects through the synthesis of phytohormones or other effectors. Past studies have shown that concentrations of cytokinins and auxins in gall-inducing insects are higher than in their host plants. However, these analyses have involved whole-body extractions. Using immunolocalization of cytokinin and auxin, in the gall inducing stage of *Eurosta solidaginis*, we found both phytohormones to localize almost exclusively to the salivary glands. Co-localization of phytohormone label with a nucleic acid stain in the salivary glands revealed the absence of *Wolbachia sp.*, the bacterial symbiont of *E. solidaginis*, which suggests that phytohormone production is symbiont independent. Our findings are consistent with the hypothesis that phytohormones are synthesized in and secreted from the salivary glands of *E. solidaginis* into host-plant tissues for the purpose of manipulating the host plant.

Keywords

Cytokinin; Auxin; Phytohormones; Insects and Gall-induction

INTRODUCTION

Plant galls are tumor-like growths of plant tissue induced by an invading organism. A wide variety of organisms are known to induce plant galls including: protists, nematodes, mites, fungi, bacteria and insects. The mechanism of induction of bacterial galls has been

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established (MacDonald et al. 1986; Lichter et al. 1995; Jameson 2000; Zhu et al. 2000; Barash and Manulis-Sasson 2007), but how insects induce plant galls remains unknown.

There are two predominant hypotheses concerning how insects induce plant galls. The phytohormone production hypothesis suggests that insects and other gall-inducing organisms induce galls by producing and secreting plant growth hormones, which lead to cell division and cell expansion forming the gall (Zhu et al. 2000; Mapes and Davies 2001a, b; Reineke et al. 2008, Dorchin et al. 2009, Straka et al. 2010, Bruce et al. 2011, Connor et al. 2012, Yamaguchi et al. 2012; Tanaka et al. 2013; Bartlett and Connor 2014; Takei et al. 2015; Chanclud et al. 2016; Kai et al. 2017; Andreas et al. 2020). Alternatively, the effector protein hypothesis suggests that gall-inducing insects and other organisms might secrete effector proteins that stimulate production of phytohormones or have effects elsewhere in the cell cycle of the host plant (Wu and Baldwin 2010; Zhao et al. 2015; Cambier et al. 2019; Zhao et al. 2019). Specific examples from bacteria and fungi illustrate that phytohormone production alone or in combination with secreted effector proteins can contribute to gall induction (Zhu et al. 2000; Barash and Manulis-Sasson 2007; Doehlemann et al. 2008; Redkar et al. 2015).

Auxins and cytokinins are classes of phytohormones that induce cell growth and cell division which could contribute to the proliferation of the plant tissues formed in interactions between insects, fungi, microbial pathogens, and plants (Bruce et al. 2011; Connor et al. 2012; Erb et al. 2012; Giron et al. 2013; Bartlett and Connor 2014; Tooker and Helms 2014; Giron and Glevarec 2014; Naseem et al. 2014; Sugio et al. 2015; Robischon 2015; Dodueva et al. 2020). Auxin, also often associated with gall-inducing insects, affects the host-plant by promoting cell elongation and maintaining apical dominance by inhibiting the formation of lateral buds (Tooker and DeMoraes 2011). Cytokinin in the presence of auxin leads to cell division and promotes the growth of plant tissues that constitute the enclosing gall (Galuszka et al. 2008; Tooker and Helms 2014).

In past studies, cytokinin and auxin have been found not only in galled plant tissue, but also in the associated gall-inducing insects at concentrations much higher than in the plant (Mapes and Davies 2001a, b; Dorchin et al. 2009; Straka et al. 2010; Yamaguchi et al. 2012; Tanaka et al. 2013; Takei et al. 2015; Kai et al. 2017; Andreas et al. 2020). The high concentrations of cytokinin and auxin in gall-inducing insect species further implicates phytohormones as playing key roles in gall induction. However, most studies have used analytical chemistry to determine whole body concentrations of cytokinins and auxin in insects, so that little is known about their distribution within the bodies of gall-inducing insects. In only one instance have the concentrations of auxin and cytokinin been estimated in the organ responsible for gall-induction. In *Pontania sp.* (Hymenoptera: Tenthredinidae), where the ovipositing female induces the gall, the accessory gland associated with the ovipositor of adult females was specifically examined for concentrations of auxin and cytokinin rather than using whole body estimates (Yamaguchi et al. 2012). Furthermore, few studies allow time for larvae that induce galls to purge their gut prior to chemical analysis which makes it difficult to separate levels of phytohormones in insect tissues from those in the plant material that remains in the gut. Therefore, it is possible for the observed levels of cytokinin and auxin in gall-inducing insects to be at least partly the result of the

consumption of plant tissues containing these phytohormones, rather than to their production by the gall-inducing insect.

Immunolocalization with commercially available antibodies has been used to examine the distribution of cytokinins and other phytohormones within galled-plant tissues (Zavala and Brandon 1983; Eberle et al. 1987; Sossountov et al. 1988; Dewitte et al. 1999; Dewitte and Van Onckelen 2001; Witiak 2006). Gall-inducing insect systems are difficult to manipulate due to insect development being closely tied to their plant host. If harm is caused to the host during experimental procedures, larval and gall development may cease. Additionally, phytohormones can also be challenging to study due their small size (~200–500 Da), relative to proteins, which prevents them from being assayed using dot blots or related molecular techniques. These aspects of phytohormones severely limit the tools available to study them, especially in a closed insect-gall system, but gives promise to immunolocalization as a viable tool for further studies. Antibodies are available for one form of auxin [indole-3-acetic acid (IAA)] and two forms of cytokinin [trans-zeatin riboside (tZR) and N-(6)-isopentenyladenosine (iPR)]. Antibodies have highly specific structural requirements for binding which renders the detection of plant hormones in tissues by immunolocalization a powerful method to study the distribution of these signaling molecules (Dewitte et al. 1999). Both tZR and iPR and their free base, nucleotide, and some methylthiol and glucoside forms, all occur at high concentrations in the gall-inducing stages of a variety of insect species, including green-island inducing moths (Mapes and Davies 2001b; Straka et al. 2010; Yamaguchi et al. 2012; Zhang et al. 2017; Andreas et al. 2020). Auxin (IAA) also occurs at high concentrations in gall-inducing insects (Mapes and Davies 2001a; Yamaguchi et al. 2012; Tanaka et al. 2013; Takei et al. 2015). Thus far, however, antibodies to cytokinin and auxin have only been used to localize these compounds in plant tissue, and not insect tissue. To our knowledge, this paper offers the first example of localization of cytokinin and auxin in the tissues of a gall-inducing insect.

Bacterial symbionts of insects may also play a role in gall induction through synthesis of phytohormones. *Phyllonorycter blancardella*, a leaf-mining caterpillar that feeds inside leaves, secretes high levels of cytokinins (Giron et al. 2007; Zhang et al. 2017) which prevents leaf tissues surrounding the leaf mine from senescing, thus forming "green islands" where the larvae feed and develop as the remainder of the leaf senesces (Angra-Sharma and Sharma 1999; Walters et al. 2008). *P. blancardella* is known to have a symbiotic *Wolbachia sp.*, and experiments with exposure of adults to antibiotics have prevented "green island" formation by their offspring (Kaiser et al. 2010; Body et al. 2013). Although *Wolbachia* is not a bacterium previously characterized to produce phytohormones, it has been implicated as the source of cytokinin production in *P. blancardella*.

To determine if phytohormones are present in insect tissues that would suggest they are delivered into the host-plant, we examined dissected tissues using immunohistochemical methods to detect cytokinins and auxins in the larvae of *Eurosta solidaginis*. *Eurosta solidaginis* is a gall-inducing fly that attacks the apical meristem of *Solidago altissima* forming a ball-shaped gall. A single bacterial symbiont, identified as a strain of *Wolbachia sp.*, has also been identified in *E. solidaginis* using 16s rRNA amplicon screening (Hammer et al. 2020). To determine if *Wolbachia* sp. in *E. solidaginis* could be responsible for these

phytohormones, we used a generic nucleic acid stain, DAPI (4′,6-diamidino-2-phenylindole), to determine if *Wolbachia* sp. and phytohormones co-localize.

METHODS

Insect and Host Plant System

We examined the distribution of phytohormones in *Eurosta solidaginis* Fitch (Diptera: Tephritidae). The biology of *E. solidaginis* and its relationship with the host plant *Solidago altissima* L. (Asterales: Asteraceae) has been extensively researched and can be considered a model system for gall induction in insects (Abrahamson and Weis 1997). *Eurosta solidaginis* is univoltine and widely distributed in eastern and mid-western North America, producing ball-shaped galls (1–4cm diameter) in the stems of its host plant *S. altissima*. Chemical analyses of *E. solidaginis* and tissues of *S. altissima* indicate that *E. solidaginis* has much higher concentrations of cytokinins and auxins than in galled or un-galled plant tissues (Mapes and Davies 2001a, b; Andreas et al. 2020). Early larval stages of *E. solidaginis* induce the gall during feeding, so the salivary glands are the most likely source of phytohormone transfer to plant tissues through herbivory (Miles 1968).

Insect Sample Collection

Galls containing *Eurosta solidaginis* (Diptera: Tephritidae) larvae were collected in Duluth, Minnesota (N 46.675871, W −92.229560) on July 24th, 2017 and July 23rd, 2018 from *S. altissima* and shipped to San Francisco on dry ice. At that time of year, 2nd instar larvae are most abundant, but only slightly larger in size than 1st instars. Galls are still actively growing at this stage of larval development (McCrea et al. 1985; Mapes and Davies 2001a, b). Galls were then stored at 4°C, until we removed larvae from the galled plant-tissue using a sterile paring knife. After we removed larvae from the gall, larvae where immediately put aside in sterile micro centrifuge tubes for dissection with full gut contents.

Insect Tissue Collection and Storage

We dissected *E. solidaginis* larvae for their primary body parts at the second instar stage. We harvested body wall (musculature and integument including exoskeleton), gut (including Malpighian tubules), and salivary glands. We placed these tissues directly into sterile phosphate buffered saline x1M (PBS). We stored the separated tissue types in micro-centrifuge tubes with small amounts of PBS and fixed tissues within two hours. Our protocol for tissue fixation and antibody staining, described below, was adapted from Barbosa et al. (2014) and Thermo Fisher Scientific Protocols (2021).

Fixation

We fixed the tissues under sterile conditions using 3% paraformaldehyde (PFA) in PBS for 30 min, followed by a second fixation step with fresh fixative solution on ice for 2.5 h. We then washed samples 3 times in PBS for 45 min for each wash and stored in PBS at 4°C until used for antibody staining.

Immunohistochemical Staining of *E. solidaginis* Tissues

We stained the dissected tissues under sterile procedures using one of three different primary antibodies: Agrisera® rabbit-anti-*N*-(6)-isopentenyladenosine [Agrisera (Vännäs, SWEDEN): Cat. No. AS09 434] (iPR), Agrisera® rabbit-anti-trans-zeatin riboside [Agrisera: Cat. No. AS09 428] (*t*ZR), and Agrisera® rabbit anti-indole-3-acetic acid [Agrisera: Cat. No. AS06 193] (IAA). To prepare tissues for antibody staining, samples were first washed with blocking buffer PBSB (PBS + 1% Bovine Serum Albumin (BSA)) 3 times, 10 minutes each wash, followed by a 30 minute wash with PBT (PBS + Triton 0.1%). The primary antibodies (anti-iPR, anti-*t*ZR and anti-IAA) were added to the samples in blocking buffer composed of PBSB at 4°C rotating for 48 h. All antibodies were diluted in PBSB at a concentration of 1:250. The specificity of these antibodies has previously been established (Dewitte et al. 1999). The cytokinin antibodies are known to be cross reactive with freebase, riboside, nucleotide, and glucoside forms of the respective hormone, but not cross reactive with forms of cis-zeatin or dihydrozeatin. The antibody for IAA is very specific to the antigen (Dewitte et al. 1999). After washing, the antibody-stained tissues were subsequently stained with a secondary goat anti-rabbit polyclonal antibody conjugated with FITC [Vector Labs (Burlingame, CA, USA): Cat. No. BA-1000] diluted 1:1000 in PBS and incubated for 18 h at 4°C rotating in the dark. After the tissues were stained with the secondary antibody, the tissues were washed five times with PBSBT (PBS + 0.1% Triton and 1% BSA) for 10 min each, to ensure removal of excess antibodies that were unattached to antigens.

Co-localization with DAPI

After washing off secondary antibody, we added DAPI (4′,6-diamidino-2-phenylindole) to antibody-stained tissues to visualize the bacterial symbiont of *E. solidaginis*, *Wolbachia* sp. (Hammer et al. 2020). DAPI is a generic nucleic acid which attaches itself to the AT regions of dsDNA, which will stain both the nucleus of the insect's cells and the chromosomes of any bacterial symbiont. DAPI diluted 1:1000 in PBS was added to the same tube in which tissues were stained and incubated for 8 min. Tissues were then washed in PBS 3 times for 10 mins each wash to ensure the removal of excess DAPI stain. We then mounted all tissues onto glass slides using VECTASHIELD® [Vector Labs (Burlingame, CA, USA): Cat. No. H-1000–10] and sealed with nail polish. After preparation, we placed slides in slide boxes at −20°C for long term storage.

Controls

We examined various controls to rule out autofluorescence and non-specific staining. To check for autofluorescence from the insect tissues, we performed a negative control which consisted of just the three insect tissues, salivary glands, gut, and body wall with buffer and washes and no antibodies, as well as a sample with primary antibody only. To test for non-specific staining, we followed the same protocol steps with only the secondary antibody. Autofluorescence was detected in the exoskeleton of the body wall, but non-specific staining with secondary antibody alone was not noted (See Supplementary Information).

Wolbachia sp. are known to exist within ovarian tissues (Werren 1997), thus we also examined ovarian tissues as a positive control for the presence of *Wolbachia sp.* using our method. Ovaries from laboratory reared adult females were dissected in July 2017 and stored

at 4°C. We fixed ovarian tissue as outlined above, but with the addition of heptane in a 1:1 ratio with PFA to help remove the chorion from ovules to facilitate penetration of the stain throughout the sample. We applied the staining protocol described above for DAPI, but with no prior antibody staining to ovarian issues. *Wolbachia* sp. have been successfully localized in other insect species in non-ovarian tissues using DAPI without the use of heptane (Karr et al. 1998; Alberston et al. 2013). However, heptane has been commonly utilized in ovarian tissues and ovules to de-chorionate ovules to allow better permeabilization to enhance visualization of *Wolbachia* sp. (Limbourg and Zalokar 1973; Müller 2008; Rand et al. 2010).

Replication

Four biological replicates of experimental tissues (primary and secondary antibody, and DAPI treated) of each tissue type and each antibody were stained and visualized with three technical replicates mounted on each slide. Controls were performed on two biological replicates with three technical replicates mounted on each slide.

Visualization/Imaging

We visualized prepared slides using a Zeiss LSM 710 Confocal Microscope (Zeiss: Oberkochen, Germany). We assessed images for localizing phytohormones within the sample tissues. To visualize co-localization of DAPI and FITC stains to the cellular level, images were acquired by taking confocal sections of each tissue sample with an EC Plan-Neofluar 40x/1.3 oil lens. All co-localized images were taken while the lasers were set for DAPI (405nm laser, 410–497 nm emission) and FITC green (488 nm laser, 493–634nm emission). Singular DAPI stained images were taken with a 405nm laser (410–585 nm emission). Images were processed using Image J (Schneider et al. 2012), and images are shown with the fluorescent label used for tagging FITC depicted in green and DAPI depicted in blue. FITC-tagged phytohormones were not manipulated for brightness or contrast, but DAPI labelled nucleic acids were enhanced with both brightness and contrast filters applied to the whole images to maximize the probability of detecting bacterial chromosomes if present.

PCR of *Wolbachia* WSP gene

Whole genomic DNA was extracted from fresh ovaries of *E. solidaginis* using the Quick-gDNA MiniPrep Kit (Zymo Research, Irvine, CA, USA). A 200bp fragment of the *Wolbachia* surface protein locus (WSP) of the *Wolbachia* strain associated with *E. solidaginis* was amplified using PCR with primers 264f (5′ - GGGCTTTATTCGCAGCTAAGC - 3′) and 463r (5′ - TGACTACTCACAGCGGTTGC - 3′). Primers were designed based on a preliminary assembly of Illumina 100bp single-end cDNA derived from a meta-transcriptomic study and assembled using ABySS (Birol et al. 2009). The target locus was amplified with 20μl PCR reactions using AccuPower PyroHotStart Taq PCR PreMix (Bioneer, Alameda, CA, USA). Each PCR reaction included 18μl PCR water, 0.5μl of each primer, and 1μl of the genomic DNA. The PCR involved an initial denaturation at 94 °C for 4 minutes, 25 cycles of denaturation (94 °C for 30 seconds), annealing (53 °C for 35 seconds), and extension (72 °C for 50 seconds), and were followed by a final extension at 72 °C for 1 minute.

RESULTS

Localization of phytohormones

All images we present are representative of the set of biological and technical replicates for each antibody and tissue type. Variation among images for each antibody/tissue type combination was minimal.

Overall, immunolocalization indicated that cytokinin *t*ZR-like and iPR-like immunoreactivity was found almost exclusively in the salivary glands of *E. solidaginis* (Fig. 1). Given the cross reactivity of the antibodies, staining may have included *t*ZR and iPR, and their free base, nucleotide and glucoside forms. iPR-like immunoreactivity localized and aggregated to the cell membrane of salivary gland cells (Fig. 1b) which is similar to its location in plants (Eberle et al. 1987), while *t*ZR-like immunoreactivity localized intracellularly within the cytoplasm of the salivary glands (Fig. 1e). The high intensity of fluorescence at each point suggests possible localization of *t*ZR to vacuoles. *t*ZR-like immunoreactivity also localized intracellularly in small quantities in gut tissue samples (see arrow in Fig. 1f). Although the stained gut tissue samples were full gut specimens, the staining was consistent with intracellular staining. iPR-like immunoreactivity was absent from full gut tissue samples (Fig. 1c).

Similar to *t*ZR, immunolocalization also suggested that auxin (IAA) localized to the cytoplasm in the salivary glands (Fig. 1h), but was mostly absent from other tissues. Salivary gland tissue showed high amounts of specific IAA-like staining intracellularly in the cytoplasm (Fig. 1h). The gut tissue showed only trace amounts of IAA-like fluorescence (arrowhead) intracellularly within the cytoplasm (Fig. 1i).

Staining was absent in the body wall for iPR (Fig. 1a), *t*ZR (Fig. 1d) and IAA (Fig. 1g), although autofluorescence was obvious in these larval body wall tissue samples. This was likely due to the chitinous composition of the outer layer of insect integument, because similar autofluorescence was absent in the gut (Fig. 1c and i), and because autofluorescence was especially noticeable in the denticles (Fig. 1a; arrowhead) and some cuticle.

Co-localization of *Wolbachia sp.*

DAPI stained the insect cell nuclei of the salivary glands, body wall, and gut tissues, as indicated by consistently large stained nuclei (blue). The lack of smaller stained components suggested that the smaller chromosomes of the bacterial symbiont, *Wolbachia sp.*, and therefore *Wolbachia* itself, were not present in these tissue types. The lack of staining of *Wolbachia* sp. chromosomes, particularly in the salivary glands where *t*ZR, iPR, and IAA localize suggests that these phytohormones do not co-localize with *Wolbachia sp.* (Fig. 1).

PCR analysis of a marker of the *Wolbachia* sp. WSP gene, indicated that the ovaries of *E. solidaginis* contain *Wolbachia* sp. Thus, we established the validity of the DAPI staining technique for detection of *Wolbachia sp.* within *E. solidaginis* tissue by staining ovary tissue. DAPI staining in the ovarian tissue is consistent with intracellular localization of a bacterium (Fig. 2), where both ovarian nuclei (large arrowheads) and smaller flecks (small arrowheads), consistent with bacterial chromosomes, were detected, confirming presence of

the strain of *Wolbachia sp.* in *E. solidaginis* by PCR. The sister strain, *Wolbachia pipientis*, has rod-like cells (0.5–1.3 μm) and coccoid forms that range widely in size (0.25–1.8 μm) (Stouthamer et al. 1999) which are similar in size to those we detected in the ovarian tissue (Fig. 2). Measurements made on the small flecks stained with DAPI (Fig. 2b), which we presume to be the chromosomes of *Wolbachia* sp., are similar in size to those reported for *W. pipientis* (mean = 0.998, se±0.05 μm, n=30). DAPI staining of the ovary was compared to that of a salivary gland sample that was stained with DAPI. There were some specks present, but they were not consistent with the size range of *Wolbachia* sp. (mean=2.06, se±0.702 μm, n=30), and Hammer et al. (2020) report no other resident bacterial symbionts for *E. solidaginis*.

DISCUSSION

Our experiments have shown that cytokinins (*t*ZR, iPR and their free base, nucleotide, and glucoside forms) and auxin (IAA) within *E. solidaginis* larvae localize predominantly to the salivary glands. Because *E. solidaginis* larvae induce plant galls, localization of phytohormones to the salivary glands is consistent with the idea that they are produced in the salivary glands and secreted into host-plant tissues for the purpose of manipulating the host plant. The antibody staining in the salivary glands implies that in *E. solidaginis*, the reported high whole-body concentrations of auxin and cytokinins (Mapes and Davies 2001a, b, Andreas et al. 2020) are almost entirely found in the salivary glands. A wide range of glandular secretions are known to be produced by insects, so that galls induced by feeding are likely to arise because of salivary secretions (Miles 1968; Doyle and Laufer 1969) and those induced by oviposition via glandular secretions associated with the ovipositor (Yamaguchi et al. 2012).

Localization of *t*ZR and IAA in gut tissue was much less prevalent than in salivary glands. The amount of staining of *t*ZR and IAA intracellularly in gut tissues was visibly much less than in the salivary glands (Fig.1f, i). While it is possible that some absorption and sequestration of *t*ZR and IAA from dietary sources occurs, the high concentrations in the insect relative to the host plant (up to 90 fold difference) suggest that this could only be a minor contribution to the overall pool of IAA and *t*ZR (Mapes and Davies 2001a, b; Straka et al. 2010, Bartlett and Connor 2014; Andreas et al. 2020). Phytohormone localization was performed on early instar larvae with full guts to gage the impact of dietary sources of phytohormones. We observed no extracellular staining of any phytohormones in the gut, implying that gut contents contributed little to the whole-body estimates of cytokinin or auxin concentrations.

It is possible that some phytohormone synthesis occurs in gut tissues. The intima of the foregut of insects is chitinous and non-secretory. However, in the midgut, digestive enzymes are synthesized and secreted, so conceivably phytohormone synthesis could occur in the lumen of the midgut. Alternatively, the cellular material in gut samples could have been of plant origin, or tissues from the salivary glands could have contaminated our whole mounts of gut and Malphigian tubules, giving the false impression that auxin and cytokinins are synthesized there as well. Therefore, we conclude that cytokinins and auxins in gut tissues are at most a very small part of the overall pool of phytohormones in *E. solidaginis*.

Our results are consistent with those reported for other members of Insecta. The gall-inducing wasp *Pontania* sp. accessory glands associated with the ovipositor of *Pontania* sp., had the highest concentrations of cytokinins ever reported for a gall-inducing insect, along with substantial concentrations of auxin (Yamaguchi et al. 2012). Stable isotope labeling has shown that nymphs of the mirid bug *Tupiocoris notatus*, which does not induce a gall, deliver cytokinin, specifically iP (isopentenyl adenine), into host-plant tissues resulting in manipulation of the host plant (Brütting et al. 2018). The presence of auxin and cytokinins in glands where they can be delivered to the host plant implies that they are produced in these glands for secretion into host-plant tissues to manipulate the host plant.

The hypothesis that a bacterial symbiont could contribute to gall induction by insects stems from two pieces of evidence. First, there are known bacterial species that induce plant galls that have biosynthetic pathways to produce auxin and cytokinin (MacDonald et al. 1986; Lichter et al. 1995; Jameson 2000; Barash and Malulis-Sasson 2007, 2009), bolstered by experiments involving *Wolbachia* sp. in the "green island" inducing *P. blancardella* (Giron et al. 2007; Kaiser et al. 2010; Body et al. 2013; Zhang et al. 2017). Secondly, by the apparent lack of biosynthetic pathways for auxin and cytokinin production in insects (Frébort et al. 2011). Our attempt to co-localize phytohormones and the *Wolbachia* sp. symbiont of *E. solidaginis* using a nucleic acid stain (DAPI) did not show staining of bacterial chromosomes in any of the larval tissues we examined, despite enhancing both the brightness and contrast for the blue wavelengths to increase the chances of detecting a signal (Fig. 1). However, we did detect bacterial chromosomes in the ovaries of adult females, indicating that our assay was effective (Fig. 2). *Wolbachia* sp. is known to reside in the reproductive organs of its host, so we interpret the absence of *Wolbachia* sp. from the salivary glands, where the phytohormones localize, to indicate that *Wolbachia* sp. does not contribute to phytohormone production in *E. solidaginis*. Although *Wolbachia* sp. in the past was implicated in cytokinin production, recent evidence further suggests that symbionts may not be contributing to phytohormone production in insects or to gall induction. An analysis of six species of gall-inducing cynipid wasps using transcriptome and whole genome sequencing, found that only two species had *Wolbachia* sp. symbionts present (Hearn et al. 2019). Hou et al. (2020) also reported multiple populations of the chestnut gall wasp, *Dryocosmus kuriphilus* (Hymenoptera: Cynipidae) that induce galls while lacking a *Wolbachia* sp. symbiont. Finally, examination of twelve insect species including gall-inducing and non-gall-inducing species using 16s rRNA amplicon sequencing yielded no evidence of a specific bacterial symbiont or a community of symbionts associated with gall induction (Hammer et al. 2020).

So, if a bacterial symbiont is not involved in the provisioning of auxin and cytokinins for gall-inducing insects, how do insects synthesize these compounds? Virtually all the research on cytokinin and auxin biosynthesis focuses on plants. Among plants, the consensus for cytokinin is that the de novo-synthesis-ipt pathway (adenylate dimethylallyl transferase, EC: 2.5.1.27 and EC: 2.5.1.112) accounts for the bulk of cytokinins found in plants, and furthermore the *t*RNA-ipt pathway leads largely to the production of cis-zeatins rather than to *t*Z or iP, which are the forms that are in high concentration in gall-inducing insects (Mapes and Davies 2001b; Straka et al. 2010; Yamaguchi et al. 2012; Tanaka et al. 2013; Takei et al. 2015; Kai et al. 2017; Andreas et al. 2020). However, the *t*RNA-ipt pathway

(tRNA dimethylallyl transferase, EC: 2.5.1.75) for cytokinin biosynthesis is found in insects and in all organisms, although previously thought be absent from Archaea (Frébort et al. 2011; Nishii et al. 2018) is has recently been detected in this domain (Wang et al. 2020). Additionally, distantly related taxa from gall-inducing fungi to dogs, have been found to contain high concentrations of cytokinins, including tZ and iP, yet only possess the tRNA-ipt pathway (Morrison et al. 2015 a, b and Seegobin et al. 2018). Cytokinins have recently been reported to be widespread and abundant in plant-feeding insects, not just in gall-inducing species, and the tRNA-ipt pathway is likely the source of these cytokinins (Andreas et al. 2020). We note that the concentrations of cytokinins in gall-inducing insects such as *E. solidaginis* are much higher than in plants, and mostly in the form of tZ and iP and their ribosides, nucleotides, and glucosides. Such a pattern would seem to undermine the notion that the tRNA-ipt pathway is unproductive and only yields cis-zeatins as claimed in the literature on cytokinin biosynthesis in plants (Sakakibara 2006; Galuszka et al. 2008; Stirk and van Staden 2010; Frébort et al. 2011; Spichal 2012). We concur with the suggestion that the tRNA-ipt pathway functions differently and considerably more efficiently in insects for the production of cytokinins than in plants (Andreas et al. 2020).

Both *Eurosta solidaginis* and *Wolbachia* sp. have the tRNA-ipt pathway, so it is still conceivable that *Wolbachia* sp. could contribute to gall induction for *E. solidaginis*. This could occur either via production of cytokinins in tissues that we did not survey, such as the fat body, and their subsequent translocation to the salivary glands, simply by their production of HMBDP which can serve as the prenyl group donor necessary for cytokinin synthesis, or by secretion of other effectors. Sorting out this possibility will require either targeted gene expression analysis or transcriptomic studies. However, the lack of any evidence of *Wolbachia* sp. in the salivary glands suggests that it is unlikely that *Wolbachia* sp. contribute to gall induction in *E. solidaginis*.

The literature on auxins reveals several pathways for biosynthesis that are found in plants and/or bacteria (Woodward and Bartel 2007; Spaepen et al. 2007; Masaguichi et al. 2011; Patten et al. 2013; Tivendale et al. 2014; Yue et al. 2014), and partial pathways defined for some fungi (Reineke et al. 2008). However, despite a few older studies reporting auxins in animals (Weissbach et al. 1959; Gordon and Buess 1967), only recent work on silkworms has attempted to elucidate an auxin pathway in animals (Suzuki et al. 2014; Yokoyama et al. 2017; Takei et al. 2018). All identified pathways for the biosynthesis of auxin involve tryptophan as the precursor, which insects freely obtain from their diet or from bacterial symbionts. However, *Wolbachia* sp. lack the ability to synthesize tryptophan so cannot be a source of tryptophan for their insect hosts (Xie et al. 2003). Therefore, auxin production by *E. solidaginis* is likely to arise from a pathway that is potentially widespread among animals.

We have built a firm case for a contribution of secreted auxin and cytokinin in the stimulation of the tissue growth in the host plant that constitutes the covering gall – the phytohormone production hypothesis. We provide strong evidence that the *Wolbachia* sp. symbiont of *E. solidaginis* is unlikely to be involved in phytohormone production and gall induction. However, the evidence we provide here for localization of auxin and cytokinin to a glandular structure primed to deliver secretions to the host plant does not rule out the possibility that secreted effector proteins also could contribute to gall induction by insects –

the effector protein hypothesis. Continued inquiry into the mechanism of gall-induction in insects, and into the production and use of phytohormones in the manipulation of plants by phytophagous insects will be required to determine the relative merits of these hypotheses to gall induction by insects.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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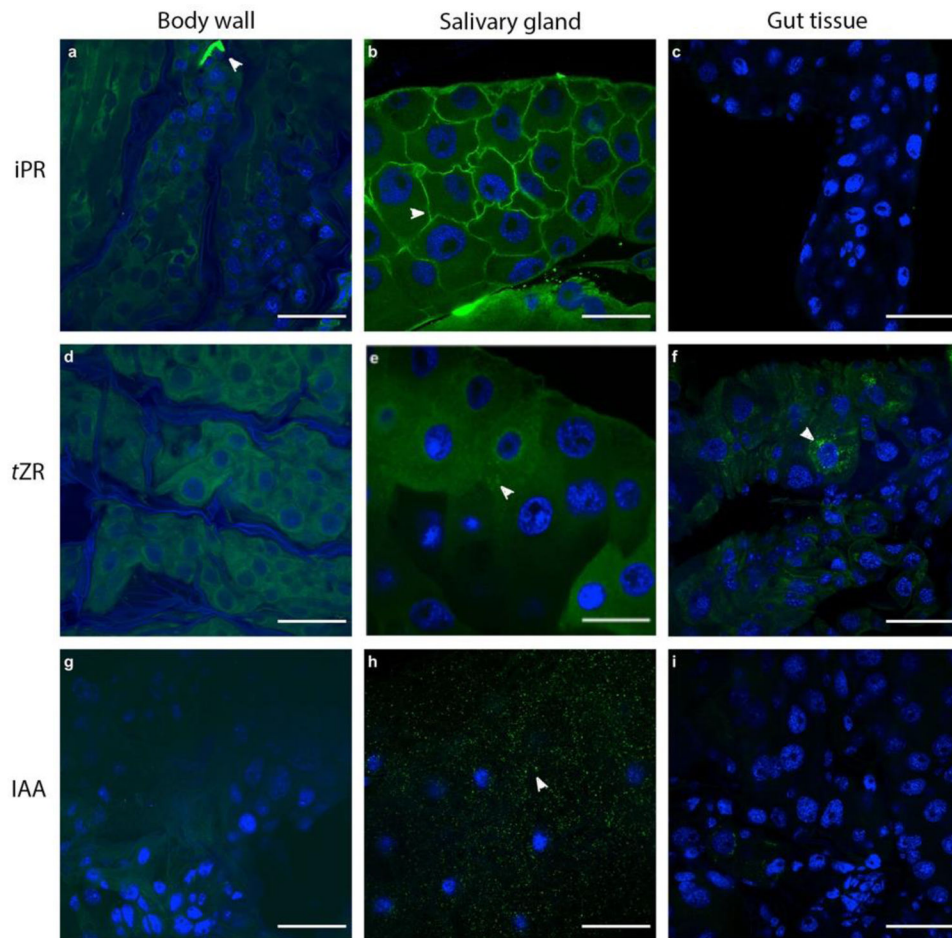
Fig 1.
Localization of cytokinin and auxin antibodies, and co-localization of *Wolbachia sp.* with DAPI nuclear stain. Immunolocalization using rabbit anti-iPR, *t*ZR and IAA antibodies in *E. solidaginis* tissues with FITC-conjugated secondary polyclonal goat anti-rabbit antibodies (green). Localization of iPR in a) body wall, b) salivary gland tissue, and c) gut tissue. Localization of *t*ZR in d) body wall, e) salivary gland tissue, and f) gut tissue. Localization of IAA in g) body wall, h) salivary gland tissue, and i) gut tissue. DAPI stain for cell nuclei and for all nucleic acids (blue) showed a lack of bacteria in these tissues based on the size of stained particles. DAPI fluorescence has been enhanced with brightness and contrast using ImageJ to identify all DAPI stain. All images were taken under 40x oil magnification. Scale bar for all images is 50 μm.
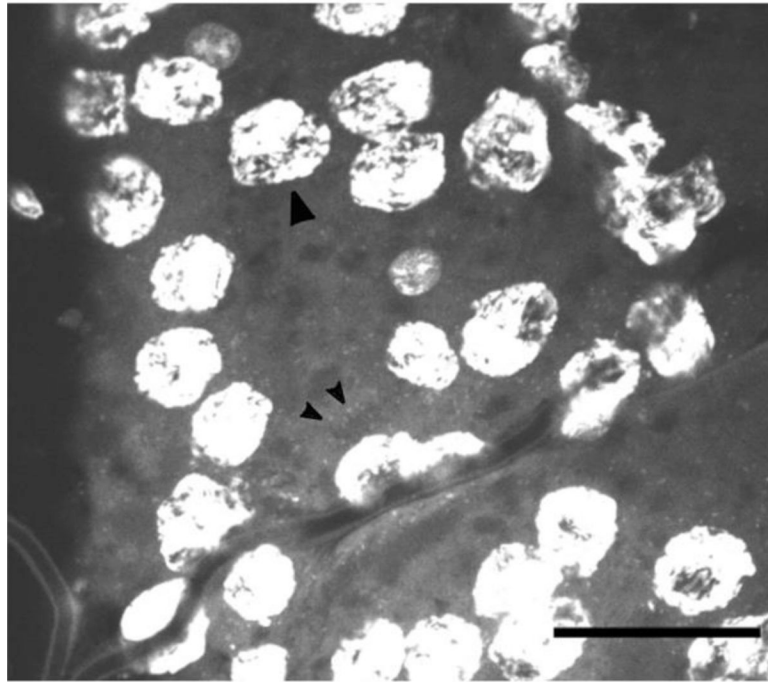
Fig 2.
DAPI stained *E. solidaginis* ovaries show cell nuclei (large arrowhead) and smaller flecks consistent with *Wolbachia* sp. chromosomes (small arrowheads). Image taken with a 40x oil-immersion lens. DAPI fluorescence has been enhanced with brightness and contrast in ImageJ to emphasize presence of small fleck staining. Scale bar for image is 50 μm.