A variety of insect species induce galls on host plants. Liquid chromatographic/tandem mass spectrometric analyses showed that a gall midge (Rhopalomyia yomogicola) that induces galls on Artemisia princeps contained high levels of indole-3-acetic acid and cytokinins. The gall midge larvae also synthesized indole-3-acetic acid from tryptophan. Close observation of gall tissue sections indicated that the larval chamber was surrounded by layers of cells having secondary cell walls with extensive lignin deposition, except for the part of the gall that constituted the feeding nutritive tissue which was composed of small cells negatively stained for lignin. The differences between these two types of tissue were confirmed by an expression analysis of the genes involved in the synthesis of the secondary cell wall. Phytohormones may have functioned in maintaining the feeding part of the gall as fresh nutritive tissue. Together with the results in our previous study, those presented here suggest the importance of phytohormones in gall induction.

Key words: gall; auxin; cytokinin; Artemisia princeps; Rhopalomyia yomogicola

Insect galls are abnormal plant tissues induced by various insect species, including wasps, midges, and aphids. The involvement of plant hormones (auxins and cytokinins) in gall formation has been suggested, not as endogenous regulators but as exogenous regulators secreted by the insects. This suggestion is based on the finding that larval parasites contained these plant hormones, sometimes at very high concentrations compared with those in the plant tissues. We have recently shown that the gall-inducing sawfly synthesized indole-3-acetic acid (IAA), an active form of auxin, de novo from a tryptophan precursor, strongly suggesting that IAA was provided to the plant tissues by the sawfly to induce the gall.1) Although we have yet to obtain direct evidence for cytokinin synthesis by the gall sawfly, we argue that it is highly probable that cytokinins are also produced by the sawfly. This argument is based on our result that female adults had a substantially high concentration of cytokinins in their gland fluid which was deposited with the egg into the plant tissue during oviposition. However, the final-instar larvae had a very low level of cytokinins and had no opportunity to acquire them from their diet before eclosion.1)

Although reports describing endogenous plant hormones in gall-forming insects have strongly suggested the involvement of these hormones in gall formation, only a few studies have analyzed these plant hormones by such reliable methods as gas chromatography/mass spectrometry (GC/MS) and liquid chromatography/tandem mass spectrometry (LC/MS/MS), using isotopic-labeled internal standards.2,3,5) Moreover, our report has been the only one describing IAA biosynthesis by a gall-forming insect.1) We focused the present study on gall midges, the largest group of gall-forming insects, belonging to the family Cecidomyiidae, to support the idea that gall-forming insects contain IAA and cytokinins at high concentrations, and that they have the capability to biosynthesize IAA. The material examined was the gall of the Japanese mugwort (Artemisia princeps) induced by the Rhopalomyia yomogicola gall midge. We analyzed and compared the endogenous levels of IAA and cytokinins in the gall midge with those in the plant tissues of the gall and in normal leaves. De novo production of IAA in the gall-forming larvae was also demonstrated in a feeding experiment.

Galls are attractive because of their characteristic visual appearance, including their various shapes and colors. A thorough understanding of the gall-inducing mechanisms makes it essential to clarify the processes underlying the characteristics of each gall. The mugwort gall is characterized by its strawberry-like shape and red color. The insect chamber is also characteristically surrounded by inflexible hard tissue, similar to woody tissue, suggesting extensive lignin deposition in the cell walls. We analyzed in this study the expression of genes putatively involved in secondary cell-wall synthesis to characterize the processes underlying the formation of this gall.

Materials and Methods

Chemicals. The stable isotope-labeled compounds, [2H5]trans-zeatin ([2H5]Z), [2H5]trans-zeatin riboside ([2H5]ZR), [6,6,2H3]isopentyl adenine ([6,6,2H3]iP), and [6,6,2H3]isopentenyl adenosine ([6,6,2H3]iPR), were purchased from OliChemIn (Olomouc, Czech Republic), [15N2]IAA was purchased from Cambridge Isotope Laboratories (Andover, MA, USA), and [15N2]-tryptophan was a...
kindly presented by Dr. Kasahara (RIKEN Plant Science Center) who purchased it from Sigma-Aldrich.

**Materials for quantitative analyses.** Galls induced by the gall midge (*R. yomogicola*) and other plant materials were collected from naturally growing Japanese mugwort (*A. princeps*). The sampling sites were the river beds of the Tsurumi River in the region of Yokohama City (Kanagawa, Japan) and Hanamuro River in the region of Tsuchiura City (Ibaraki, Japan). The galls were found between April and November, but in those found in or after August, the gall midges were usually parasitized by wasps (not identified). Only galls without parasitic wasps were used for the experiments. The leaves with galls were detached and kept cool with a refrigerant until the sampling had been completed. The galls were dissected to separate the larva and the plant tissues. Leaves without galls were used as the control tissue. The galls were grouped according to the size of the larvae, into small galls containing first- and second-instar larvae and medium galls containing third-instar larvae. About 50 mg of leaf and gall tissues and 1–2 mg of insect tissues were analyzed.

**Histological observations.** Whole galls containing larvae were fixed in an FAA solution (5% [v/v] formaldehyde, 50% [v/v] ethanol, and 5% [v/v] acetic acid), dehydrated by passage through a graded series of water–ethanol–terr-butyl alcohol, and embedded in paraffin. Sections (10 µm thick) were placed on glass slides, deparaffinized in xylene, and hydrated by passage through an alcohol series. To stain the lignin in the sections, a phloroglucinol solution (25 mg of phloroglucinol dissolved in 3.0 mL of 50% methanol, to which 1.5 mL of concentrated HCl had been added) was dropped on to each deparaffinized section. A 0.2 mL of water–ethanol–tert-butyl alcohol mixture as the template and with the following degenerates primers: ApACT1, 5'-GAGAARAGTACGAAGATY-3' sense and 5'-TCDGGTKAKRGTACGCCCG-3' antisense; ApGAPDH1, 5'-TFAAGAAGCGTCTTCCTGAC-3' sense and 5'-TCTGCTGGTTCTCCTGGT-AGTG-3' antisense; ApMYB1, 5'-TGAARMAAGGGCCGTCCTG-3' sense and 5'-ATTTGCTTGTCGTTCTCCCGG-3' antisense; and ApCAD1, 5'-TAYCCGTATGGTYCCWGG-3' sense and 5'-ACAGTGYACRGTGTDCCG-3' antisense. The amplified cDNA fragments were cloned into the pGEM-T Easy vector (Promega, Madison, WI, USA), and the sequences of the cloned fragments were determined and deposited in the DDBJ data bank under the following accession numbers: AB811625 (ApACT1), AB811627 (ApGAPDH1), AB811628 (ApMYB1), and AB811626 (ApCAD1). The PCR primers for real-time RT-PCR were prepared based on the following sequences: ApACT1, 5'-CTTCAATGTTCCTGCTATGT-3' sense and 5'-TAGATGGGGTGCTATGGGGA-3' antisense; ApGAPDH1, 5'-TGTGTTCAAGGTTTCAACAGAGCAGGA-3' sense and 5'-GGCTTCATACACACACACAGAAGA-3' antisense; ApMYB1, 5'-TGTTGTITTGAGGCGTCGTG-3' sense and 5'-TGGCAAGGTAGCTATGGAAGA-3' antisense; and ApCAD1, 5'-GACGAAGTGCCACACTACTATG-3' sense and 5'-GAATCTCCCTTTTACGCCACACT-3' antisense. Real-time (RT)–PCR was performed with a TP800 Dice Thermal Cycler real-time system (Takara) with a SYBR Premix Ex Taq mixture (Takara), by 40 cycles of 95 °C for 5 s and 60 °C for 20 s. The relative amounts of the transcript for each gene were calculated by the crossing-point method, using a standard curve according to the manufacturer’s instructions. All primer sets used for cDNA amplification were tested by a dissociation curve analysis and verified for the absence of non-specific amplification. The transcript levels of the analyzed genes were standardized by comparing with the levels of the ApACT1 transcript.

**Feeding experiments.** Three first-instar larvae were briefly rinsed with sterile water, soaked in 5 µL of water containing 2 µg of [13C5]tryptophan, and incubated at 25 °C for 15 h under dim light (7–10 µmol m−2 s−1). The incubation mixture containing the larvae was spiked with 1 ng of [13C6]IAA, extracted, purified, and analyzed by LC/MS/MS, as described by Yamaguchi et al. 

**Real-time RT-PCR analysis.** An examination of several procedures for preparing high-quality RNA with a high yield led us to the conclusion that the phenol extraction method would be suitable for normal leaf tissue of the Japanese mugwort, and that the cetyltrimethylammonium bromide (CTAB) method would be suitable for the gall tissue. Briefly, 20–40 mg of leaf tissue was homogenized in liquid nitrogen, suspended in a phenol solution (a mixture of 2 mL of TE-saturated phenol, 20 µL of 10% SDS, 87.2 mg of sodium acetate, and 40 µL of 0.5 M EDTA at pH 8.0), and extracted three times with chloroform. The resulting aqueous phase was mixed with 0.6 volumes of isopropyl alcohol and left for 10 min at ambient temperature. The precipitate was recovered by centrifuging for 10 min at 22,000 x g and 4 °C, dissolved in RNase-free water, and further purified with a total RNA extraction plant mini kit (RBC Bioscience, Tokyo, Japan). The gall tissue (20–40 mg) homogenized in liquid nitrogen was transferred to a tube containing 500 µL of a CTAB solution (2% CTAB, 0.1 M Tris–HCl at pH 8.5, 20 mM EDTA, 1.4 M sodium chloride, and 2% 2-mercaptoethanol). The suspended mixture was incubated at 65 °C for 10 min and extracted three times with an equal volume of chloroform. The aqueous phase was mixed with half a volume of 0.8 M sodium citrate containing 1.2 M sodium chloride, and a 1/4 volume of isopropyl alcohol. After incubating at −20 °C for more than 1 h, the precipitate was recovered by centrifuging for 30 min at 22,000 x g, and 4 °C. The recovered RNA fraction was dissolved in 300 µL of RNase-free water, and a 1/4 volume of 10 M lithium chloride was added. After incubating for 2 h at −20 °C, the RNA was recovered by centrifuging for 10 min at 22,000 x g and 4 °C. After the RNA had been treated with DNaseI, first-strand cDNA was synthesized from it with ReverTra Ace reverse transcriptase (Toyobo, Tokyo, Japan) according to the manufacturer’s instructions.
1 × Denhardt’s solution, 10% dextran sulfate, 300 mM NaCl, and 0.1 mM EDTA). The probe solution was incubated for 5 min at 80 °C and poured on to the pretreated sections which were covered with Parafilm (Pechiney Plastic Packaging, Menasha, WI, USA) and incubated in the dark for 16 h at 50 °C in a 50% formamide-saturated box.

After hybridization, the sections were washed three times with 4 × saline sodium citrate (SSC) for 10 min at 50 °C, treated at 37 °C for 30 min with 20 μg/mL of RNase A in NE II (10 mM Tris–HCl at pH 8.0, 500 mM NaCl, and 1 mM EDTA), and then washed three times with NE II at 37 °C for 15 min and twice with 0.5 × SSC at 50 °C for 20 min. The solution was replaced with buffer 1 (100 mM Tris–HCl at pH 7.5 and 150 mM NaCl) for the subsequent blocking procedure. The sections were treated with a 0.5% (w/v) blocking reagent (Roche Diagnostics) in buffer 1 for 1 h, then with 1% (w/v) bovine serum albumin in buffer 1 containing 0.3% (v/v) Triton X-100 for 1 h (buffer 2), 1000-fold-diluted anti-DIG AP Fab (Roche Diagnostics) in buffer 2 for 1 h, and finally in buffer 2 for 5 min. The slides were washed with buffer 1 for 5 min and with buffer 3 (100 mM Tris–HCl at pH 9.5, 100 mM NaCl, and 50 mM MgCl₂) for 5 min, and then incubated in a coloring reagent (a 50-fold-diluted NBT-BCIP solution [Sigma Aldrich Japan, Osaka, Japan]) in buffer 3). The coloring reaction was stopped after 2 h by incubating in 50 mM EDTA at pH 8.0. The sections were covered with 80% glycerol and observed under a BX-8100 microscope (Keyence, Tokyo, Japan).

Results

Observation of the gall tissues and parasitizing gall midge

The Japanese mugwort gall that is induced by the *R. yomogicola* gall midge is called ‘yomogi-ka-eboshifushi’ in Japanese according to the nomenclature that combines the host plant name, the gall site, and the appearance of the gall (such as its shape and/or color), and fushi; i.e., ‘yomogi’ means Japanese mugwort, ‘ha’ means leaf, ‘eboshi’ means a type of hat found in ancient Japan, and ‘fushi’ means insect gall. Figure 1a and b respectively show the appearance of this gall in its early and late stages. In most cases, the gall developed on the adaxial side of the leaf, and all galls were formed along secondary veins. Figure 1c–e respectively show the first-, second- and third-instar larvae, and Fig. 1f shows a pupa. The larvae were positioned in the basal part of the larval chamber at all stages (note that in Fig. 1c, the first-instar larva had been moved slightly from its original position during dissection).

The tissues of the different-stage galls were analyzed by microscopic observation of the gall sections. The gall tissue in the early stage, containing a first-instar larva, was predominantly composed of small cells (Fig. 1g and h). In the later-stage galls, the cells of the outer tissue had expanded (indicated by arrows), whereas those of the basal portion (indicated by arrowheads), where the larvae always occurred, continued to be compact (Fig. 1i–l). This suggests that this part of the tissue with compact cells was the larval-feeding nutritive tissue. As already mentioned, inflexible wood-like tissue characterizes the mugwort gall. The gall tissue breaks sharply when the gall is pinched, and it is therefore considered to contain cells with highly developed secondary cell walls. The staining of lignin, the major component of the secondary cell walls, with phloroglucinol confirmed that lignin was heavily deposited on the inner side of the gall tissue (Fig. 1m and n). Longitudinally sliced thin sections showed that lignin was first deposited in the early stage of gall formation (Figs. 1o and p), and accumulated further in the later stage (Figs. 1q and r).

It should be noted that the basal part of the gall, where the larvae were attached (indicated with arrowheads in Figs. 1p and r), was not stained with phloroglucinol, further supporting the notion that this part constituted the fresh tissue suitable for feed. Observation of the transverse sections confirmed that the inner tissue was composed of cells with extraordinarily thick cell walls (Figs. 1s–w). Sections containing the nutritive tissue again showed no cells that were stained with lignin (indicated with an arrowhead in Fig. 1w). Xylem vessels were also visualized after staining with phloroglucinol (Figs. 1x and y). The leaf vascular bundles (indicated with an arrow in Fig. 1x) were connected to the gall tissue by newly developed xylem vessels that were characterized by helical thickening (Fig. 1y, and shown in the rectangle in Fig. 1x). This vascular development is indicative of the action of auxins.²⁰

Analysis of IAA and cytokinins

The endogenous levels of IAA and cytokinins in the normal leaves, gall tissues, and larvae inside the galls were compared. The concentrations of IAA in the gall tissues and larvae did not differ significantly between the early and late developmental stages (Fig. 2). However, the IAA concentration in the gall tissue was approximatively twice as high as that in the normal leaves, and the concentration in the larvae was more than 35 times higher than that in the normal leaves. Although the concentrations of cytokinins varied more widely between samples than those of IAA, there was a clear tendency for the larvae to contain a higher concentration of cytokinins than the plant tissues.

Synthesis of IAA from tryptophan in *R. yomogicola larvae*

We next examined whether IAA detected in the larvae had been synthesized by the larvae. According to the method used in our previous study,¹¹ the larvae were soaked in a solution containing [¹⁵N]IAA production was analyzed by LC/MS/MS. [¹³C₆]IAA was clearly detected, together with endogenous IAA and [¹⁵N]IAA added after incubation as the internal standard (Fig. 3).

Expression analysis of the genes involved in secondary cell-wall synthesis

Microscopic observations (Fig. 1) showed the gall tissue to be rich in cells with highly developed secondary cell walls. To confirm this property of the gall tissue, the genes putatively involved in secondary cell-wall development were cloned from *A. princeps* and their expression was analyzed. We first chose for analysis an MYB transcription factor involved in secondary cell-wall formation. A model of the transcriptional network of secondary cell-wall biosynthesis in *Arabidopsis thaliana* has been established.²⁷,²⁸ Many MYB family members are involved in this network, some acting either positively or negatively specific in lignin synthesis, and others acting not only in lignin synthesis, but also in the synthesis of such other cell-wall elements as hemicellulose and...
cellulose. We initially attempted to clone orthologues of the *Arabidopsis* MYB transcriptional regulators, MYB46 and MYB83, which encode master switches of the secondary wall function. However, PCR-based cloning from *A. princeps* cDNA generated an MYB, designated ApMYB1, with highest homology to *Arabidopsis thaliana* MYB43 (AtMYB43). Since MYB43 has been identified as one of the direct targets of MYB46 and MYB83, and is specifically expressed in the cells in which secondary walls form,9,10) we decided to analyze the expression of ApMYB1 as a possible marker of secondary cell-wall formation in *A. princeps*. Cinnamyl alcohol dehydrogenase (CAD) was next chosen as an enzyme involved in the biosynthesis of lignin, because lignin is the most important element of the secondary cell wall, conferring rigidity to the plant. This enzyme catalyzes the reduction of hydroxycinnamaldehydes to hydroxycinnamyl alcohols, the monomeric precursors of polymeric lignin. PCR-based cloning of the CAD genes using degenerate primers produced a clone that was highly homologous to the CAD genes of several plant species, including *AtCAD4*, whose encoded CAD function has been demonstrated.11)

The results of RT–PCR showed that both ApMYB1 and ApCAD1 were upregulated in the gall tissue when compared with their expression in the control leaf tissue (Fig. 4), suggesting that the inflexible gall tissue composed of cells with thick cell walls actively produced the secondary cell-wall components, including lignin. *In situ* hybridization was performed to correlate this expression with the specific tissue types. An antisense ApMYB1 probe gave a strong blue–violet signal (Fig. 5a) in sections of a young gall, whereas the sense probe did not (Fig. 5b), confirming the specific detection of ApMYB1 gene expression (Fig. 5a). The same probe gave a positive signal in the vascular bundles, including the xylem and fiber cells of the leaf veins (Fig. 5c, arrowhead), supporting the idea that ApMYB1 would be a good marker gene for detecting secondary cell-wall formation. Close observation of the young gall clearly showed that ApMYB1 was not expressed in tissue that probably was the nutritive tissue for the gall midge larvae (Fig. 5d, arrowhead). The stained portion around the unstained tissue was composed of thick-walled cells. The older galls showed results similar to those of the young galls (i.e., there was
The validity of this probe was confirmed by the signal being specific to fiber cells in the leaf vascular bundles (Fig. 5h). Although a faint blue–violet signal was apparent in the old gall tissue (Fig. 5j), the signal intensity did not clearly differ from that produced with the sense probe (data not shown).

**Discussion**

We commenced our study with a close observation of yomogi-ha-eboshi-fushi, an A. princeps gall induced by the R. yomogicola gall midge. We first found that, at all larval stages, the larvae were positioned at the bottom of the gall. In a willow gall induced by a sawfly, which is another of our research materials, the sawfly larvae constantly move inside the galls and non-specifically eat any part of the inner callus-like tissue which could explain the concentric structure of the galls. In contrast, the structure of the A. princeps gall showed polarity from the base to the top of the gall which could be defined by the fixed larval feeding site. The feeding tissue also showed unique characteristics in comparison with the other parts of the gall: the tissue was composed of small cells and was not stained with phloroglucinol, this being indicative of extensively proliferating tissue. Several previous studies on willow galls have shown that the inside of the gall tissue was callus-like with extensive cell division, this being confirmed by the high transcript levels of cyclin D genes. The plant hormone analysis in the present study showed that the larvae contained high concentrations of cytokinins and IAA. Since these hormones promoted the division of plant cells, it is probable that the auxin and cytokinins secreted by the larvae maintained a feeding site composed of compact cells as fresh nutritive callus-like tissue. Microscopic observations showed that the xylem vessels were newly

**Fig. 2.** Concentrations of IAA and Cytokinin in A. princeps Control Leaves (L), in Gall Tissues of Small Galls (SG) and Medium Galls (MG), and in Larvae Recovered from Small Galls (SM) and Medium Galls (MM). The values above the bars indicate the plant hormone concentration. Asterisks indicate significant differences between the indicated datasets at *p < 0.05 or **p < 0.01 by Welch’s t test. Error bars are SD, n = 3.

**Fig. 3.** Mass Chromatograms of [13C10,15N1]IAA Produced from [13C15N2]-tryptophan. Gall midge larvae were incubated with [13C15N2]-tryptophan. After incubation, the reaction mixture was spiked with [13C0]IAA and pre-purified in solid-phase extraction columns, and the produced [13C10,15N1]IAA, endogenous IAA, and the [13C6]IAA internal standard were detected in the multiple reaction monitoring mode of the LC/MS/MS analysis.

**Fig. 4.** Transcript Levels of ApMYB1 and ApCAD1 in Leaf and Gall Tissues. Total RNA was prepared from leaves (L), small galls (SG), and medium galls (MG), and subjected to RT–PCR analysis. The transcript levels were standardized by comparing with the levels of the ApACT1 transcript. Asterisks indicate significant differences between the indicated datasets at ***p < 0.01 by Welch’s t test. Error bars are SD, n = 3.

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no staining in the innermost tissue at the base of the galls which was surrounded by thick-walled cells with a positive signal), except that the unstained tissue became thinner and the intensity of the signal became weaker than that in the young galls (Fig. 5e). An in situ hybridization analysis with the ApCAD1 antisense probe showed that gene expression was localized to the portion surrounding the feeding nutritive tissue (Fig. 5f and i).
The actions of auxins. We have previously observed in insect galls, and is considered to be one of xylem. Vascular development has been frequently induced to grow toward the gall tissue from the leaf veins, and the arrow in d indicates unstained tissue which was probably larval feeding tissue. Bars: 300 μm in a, b, e–g and j; 100 μm in c, d, h and i.

Fig. 5. Localization of ApMYB1 and ApCAD1 Transcripts in Leaf and Gall Tissues by in Situ Hybridization.

The expression of ApMYB1 and ApCAD1 was analyzed in longitudinal sections of an early-stage gall (ApMYB1: a, b and d; ApCAD1: f, g and i), late-stage gall (ApMYB1: c; ApCAD1: j), and leaf (ApMYB1: c; ApCAD1: b). d and i are higher magnifications of a and f, respectively. Each section was probed with the antisense (a, c–f and h–j) or sense (b and g) DIG-labeled RNA probe. The arrows in c and h indicate fiber cells of leaf veins, and the arrow in d indicates unstained tissue which was probably larval feeding tissue. Bars: 300 μm in a, b, e–g and j; 100 μm in c, d, h and i.

induced to grow toward the gall tissue from the leaf xylem. Vascular development has been frequently observed in insect galls, and is considered to be one of the actions of auxins. We have previously demonstrated the biosynthesis of IAA by the gall-inducing sawfly. We have shown in the present study that the gall midge also synthesized IAA from tryptophan. This results supports the idea that gall-inducing insects synthesize and utilize IAA for gall induction, presumably to induce cell proliferation and vascular-bundle development. Although the levels of IAA and cytokinins in the gall midge larvae were clearly higher than those in the plant tissues, the detected levels were not especially high when compared to those of other gall-inducing insects. The concentrations of plant hormones have widely varied among gall-inducing insect species. This could be attributable to the different processes of gall induction from gall to gall, and the varied sensitivity to plant hormones among host plant species.

It is also a subject of future study to elucidate the biosynthetic mechanisms of IAA and cytokinins. Although IAA has been shown to be de novo synthesized from tryptophan in larvae, it remains to be established whether larvae produce IAA with enzymes encoded by their own genome or with the help of bacterial symbionts. These possibilities are also the case for cytokinin synthesis. The identification of biosynthetic enzymes will be the next important step to fully elucidate the role of plant hormones in gall induction.

It has been suggested in previous studies that auxins and cytokinins could commonly play important roles in the formation of different types of galls. A common feature of all galls is that they are formed by abnormal cell division and differentiation, and that there is extensive vascular bundle development in gall tissues. These common properties are explicable by the activities of auxins and cytokinins. In contrast, each gall has its own distinguishing characteristics, such as its shape and color, which make galls attractive as biological phenomena. We focused this study on the formation of the secondary cell wall as a specific characteristic of the mugwort gall. The larval chamber is surrounded by hard woody tissue, and a microscopic analysis has shown this tissue to be composed of cells with thick extensively developed cell walls. Staining also confirmed that this hard tissue consisted of cells with extensive lignin deposition. The analysis of secondary cell-wall development is therefore one of the key steps in understanding the formation mechanism of the mugwort gall. In the present study, ApMYB1 encoding an MYB transcription factor and ApCAD1 encoding lignin biosynthetic enzyme CAD were cloned from A. princeps as probable candidate genes with which to monitor secondary cell-wall development. Their clear expression in vascular bundles, including xylem and fiber cells, which are typical lignin-deposited cells, confirms the relevance of these genes in this analysis. The higher expression of these genes in gall tissue than in leaf tissue supports the proposition that lignin synthesis is promoted by larval stimuli. The localized expressions of these genes roughly coincided with the localized staining of lignin with phloroglucinol, indicating the involvement of ApMYB1 and ApCAD1 in lignin synthesis in the gall tissue. The larval feeding tissue, which was not stained by phloroglucinol, showed no expression of either gene. This result further supports the idea that the feeding site is maintained as fresh nutritive tissue by repressing lignification. Given that the feeding site had a callus-like property, cytokinins and/or auxins might be indirectly involved in this repressive mechanism.

It is well known that an elicitor secreted by pathogenic fungi induces the production of so-called ‘defense lignin’ at the infection sites in plant tissues. In that case, lignin formation is considered to be a plant defense response against fungal infection. If this is also the case for the mugwort gall, the gall midge must secrete an unknown elicitor to induce lignin synthesis. ApMYB1 and ApCAD1 could be good marker genes with which to monitor this elicitor activity.
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