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Research

The airborne herbivore-induced plant volatile indole is converted to benzoxazinoid defense compounds in maize plants

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Summary

• Herbivore-induced plant volatiles act as danger signals to prime defense responses in neighboring plants, yet in many cases the mechanism behind this priming is not known. Volatile signals may be recognized directly by receptors and/or converted into other active compounds. Here we investigate the metabolic fate of volatile indole, a known priming signal in maize (Zea mays), to determine if its conversion to other compounds could play a role in its priming of defenses.

• We identified benzoxazinoids as major products from volatile indole using heavy isotope-labeled volatile indole and Pathway of Origin Determination in Untargeted Metabolomics (PODIUM) analysis. We then used benzoxazinoid biosynthesis maize mutants to investigate their role in indole-mediated priming.

• Labeled volatile indole was converted into DIMBOA-glucoside in a bx2 (benzoxazinone synthesis2)-dependent manner. The bx2 mutant plants showed elevated green leaf volatile (GLV) production in response to wounding and Spodoptera frugiperda regurgitant irrespective of indole exposure.

• Thus, volatile indole is converted into benzoxazinoids, and part of its priming mechanism may be due to the enhanced production of these phytoanticipins. However, indole-mediated enhanced GLV production does not rely on the conversion of indole to benzoxazinoids, so indole also has other signaling functions.

Introduction

Plants that come under attack from external threats such as wounding or herbivory release a bouquet of stress-related volatile organic compounds. These compounds can act as a direct defense mechanism via anti-herbivore, repellent, and anti-microbial activity. They can also aid in indirect defense by attracting parasitoids or predators, or as a priming signal to neighboring plants and distal tissues of the same plant (Sharifi et al., 2018; Hammerbacher et al., 2019; Hu et al., 2021; Ninkovic et al., 2021). Priming signals act as an alarm, preemptively inducing relevant defenses in neighboring plants or distal tissues to prepare them for a possible imminent attack (the 'primed state') (Conrath et al., 2006; Frost et al., 2008; Hu, 2021; Hu et al., 2021). These de novo defenses can include phytoalexins, toxins, and activated signaling pathways.

There is still a great deal of mystery surrounding how volatiles act in priming plant defenses, including, in many cases, how priming volatiles are perceived (Loreto & D'Auria, 2022; Wang & Erb, 2022; Kessler et al., 2023). Some volatiles are perceived directly via binding to specific receptors. The plant hormone ethylene, reviewed in Binder (2020), is a classic example of this. Other volatile receptors include TOPLESS-like proteins in tobacco that are b-caryophyllene-binding transcriptional co-suppressors, thought to be involved in volatile perception (Nagashima et al., 2019), and the karrikin-insensitive receptor (PhKAI2ia) in petunia, that perceives the sesquiterpene volatile compound (−)-germacrene D (Stirling et al., 2024). However, to date none of these perception mechanisms have been clearly linked to plant defense priming by volatiles.

It has been hypothesized that priming signals such as green leaf volatiles (GLVs), indole, and terpenoids may influence plant-to-plant signaling through their ability to dissolve in membranes and alter plant plasma membrane potentials (Maffei et al., 2001; Zebelo et al., 2012; Erb et al., 2015; Kessler et al., 2023). The electrical signals generated by these ionic membrane potential changes could trigger signaling responses in the plant. This hypothesis, however, cannot fully account for the compound specificity observed in volatile-mediated priming (Moreira et al., 2016).

Alternatively, some volatile signaling compounds are not perceived directly by receptors, rather they are taken up into plant tissues and biochemically converted into their perceived signaling forms. For example, the volatile signal methyl salicylate is

converted into the plant defense hormone salicylic acid by a methyl salicylate esterase (Forouhar et al., 2005; Gong et al., 2023). An equivalent mechanism is observed for the volatile signal methyl jasmonate, which is converted into jasmonic acid (JA) by a methyl jasmonate esterase and conjugated to isoleucine to form the hormone JA-isoleucine (Wu et al., 2008). Interestingly, in tomato, the wound-induced GLV, (Z)-3-hexen-1-ol, can be taken up by neighboring plants and glycosylated to form the anti-herbivore defense compound (Z)-3-hexenyl-vicianosid (Sugimoto et al., 2014), suggesting that priming could also involve the conversion of priming compounds into defensive compounds.

Maize (Zea mays) produces a range of volatiles following herbivory by caterpillars like Spodoptera frugiperda (fall armyworm) including GLVs, phenolics, terpenes, and indole (Frey et al., 2000; Block et al., 2018; Yactayo-Chang et al., 2024). Of these herbivore-induced volatiles, both GLVs and indole have been shown to both individually and synergistically prime neighboring maize plants; however, the mechanism behind this plant-to-plant communication is not yet known (Engelberth et al., 2004; Erb et al., 2015; Hu et al., 2018).

Volatile indole is produced in maize from indole-3-glycerolphosphate (IGP) by the enzyme indole glycerol phosphate lyase (IGL1) (Frey et al., 2000). Maize has two additional IGL isoforms that also make indole. The first, and likely ancestral enzyme, is the tryptophan synthase alpha subunit 1 (TSA1) (Kriechbaumer et al., 2008). The second IGL homolog is benzoxazinless 1 (BX1) (Frey et al., 1997) that catalyzes the first step in the synthesis of benzoxazinoids (Fig. 1). The BX1-produced indole is then converted into indolin-2-one by the indole-2-monooxygenase (BX2 – benzoxazinone synthesis 2). Mechanistically diverse BX enzymes catalyze the subsequent steps in benzoxazinoid production making a range of these glycosylated hydroxamic acids including DIMBOA-Glc (2,4-dihydroxy-7-methoxy-2H-1,4-benzoxazin-3(4H)-one-b-D-glucopyranose), HDMBOA-Glc (2-hydroxy-4,7-dimethoxy-1,4-benzoxazin-3-one-b-D-glucopyranose), and HDM_2BOA-Glc (2-2-hydroxy-4,7,8-trimethoxy-1,4-benzoxazin-3-one-b-D-glucopyranose). Benzoxazinoids have documented roles in plant defense against biotic threats, in allelopathic interactions with other plants, and in defense signaling, as well as roles in micronutrient uptake and protection from heavy metals (Niemeyer, 2009; Ahmad et al., 2011).

Treatment of the maize bx1 mutant with pharmacological levels of indole in liquid form has shown that exogenous indole can rescue benzoxazinoid production in this mutant (Frey et al., 1997; Melanson et al., 1997), raising the intriguing possibility that the production of volatile indole in stressed plants may lead to its uptake into the leaves of neighboring plants and conversion into benzoxazinoids. Moreover, the existence of multiple pathways in maize that use indole as an intermediate, indicates that volatile indole may be taken up and incorporated into several maize biosynthesis pathways, leading to the question of which, if any, of these pathways does volatile indole enters and to what extent? Finally, if volatile indole is converted into other compounds in maize leaves, do these compounds play a role in mediating indole-based priming? Multiple indole-derived compounds could potentially mediate defense signaling, including benzoxazinoid derivatives, and the phytohormone IAA.

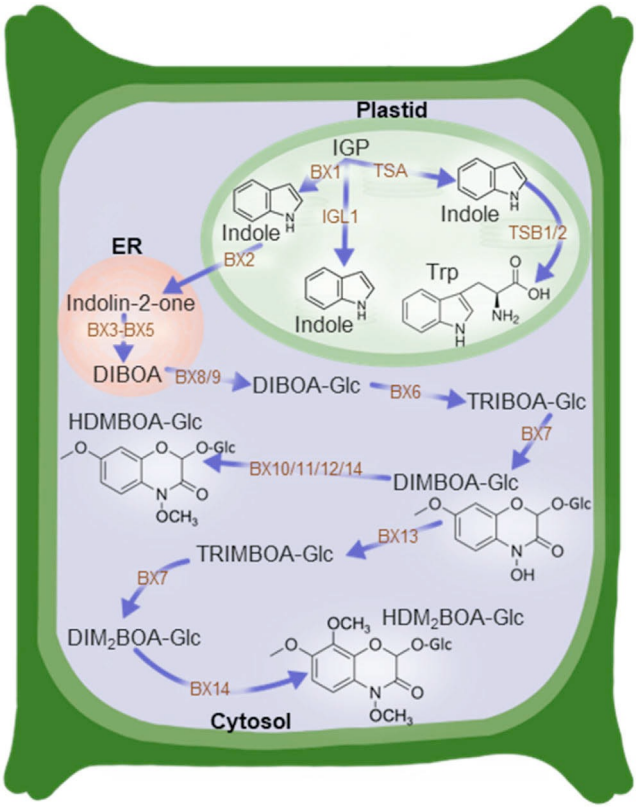

Fig. 1 Biosynthesis pathways in maize involving indole. Maize has three enzymes that make indole from indole-3-glycerolphosphate (IGP). Volatile indole is made by indole glycerol phosphate lyase (IGL1). The tryptophan synthase alpha subunit 1 (TSA1) makes indole as an intermediate in tryptophan (Trp) biosynthesis. TSA1 forms a complex with the tryptophan synthase beta subunit (TSB) channeling the indole. Indole is also made by the IGL1 homolog benzoxazinless 1 (BX1) as the first step in benzoxazinoid biosynthesis. This indole is then converted into indolin-2-one by the indole-2-monooxygenase (BX2 - benzoxazinone synthesis 2). Mechanistically diverse BX enzymes catalyze the subsequent steps in benzoxazinoid production making a range of these glycosylated hydroxamic acids including DIMBOA-Glc (2,4-dihydroxy-7-methoxy-2H-1,4-benzoxazin-3(4H)-one-b-D-glucopyranose), HDMBOA-Glc (2-hydroxy-4,7-dimethoxy-1,4-benzoxazin-3-one-b-D-glucopyranose), and HDM_2BOA-Glc (2-2-hydroxy-4,7,8-trimethoxy-1,4-benzoxazin-3-one-b-D-glucopyranose), ER, endoplasmic reticulum.

In this study, we use heavy isotope-labeled volatile indole, untargeted and targeted metabolite analysis, and maize biosynthetic mutants to investigate these questions.

Materials and Methods

Plant material

The bx1::Ds loss-of-function maize (Zea mays L.) plants carry the Dissociator (Ds) transposon insertion in ZmBX1 (GRMZM2G085381) in the maize inbred line W22 (Tzin

et al., 2015). Their corresponding wild-type is W22 (r-sc::m3). The bx2-umu1 loss-of-function mutant carries a Mutator (Mu) transposon in the second exon of ZmBX2 (GRMZM2G085661). The transposon allele of ZmBX2 (bx2-umu1) was identified in UniformMu (Settles et al., 2007; McCarty et al., 2013) family UFMu-07281, which contained the insertion mu1056648 within the coding sequence of the second exon of ZmBX2. Seeds obtained from the Maize Genetic Cooperation Stock Center (Urbana, IL, USA) were grown and the plants were analyzed for the presence of the transposon by PCR using gene-specific primers (5′-GGTGCTGTAAATGCCCTAACAATCT-3′ and 5′-GACGAGGACGACGATAA-GGACTTCA-3′) along with a Mutator-specific terminal inverted repeat primer TIR6 (5′-AGAGAAGCCAACGCCAWCGCCTCYATTTCGTC-3′). Conditions for all PCR reactions followed protocols from Phusion high-fidelity DNA polymerase (NEB, Ipswich, MA, USA; Cat. M0530L). Seed from self-pollinated ears of homozygous plants were used as the source for the bx2-umu1 mutant. The corresponding wild-type for bx2-umu1 is bzW22.

Labeling with volatile heavy-isotope indole

Plants were grown in a glasshouse under supplemental lighting in individual pots and at 3 wk old was transferred to glasshouse-located volatile collection chambers (volatile collection system model TVCSx4V; Analytical Research Systems, Gainesville, FL, USA) with an incoming airflow of 1.7 l min^{-1} and a vacuum of 0.7 l min^{-1}. Labeling was performed with unlabeled indole (Sigma-Aldrich) or heavy isotope indole ($^{13}C_8$;^{15}N) labeled with ^{13}C on all carbons and ^{15}N on its nitrogen with a chemical purity of ≥ 98% and an isotopic purity of $^{13}C8$ 98%; ^{15}N, 96–99% (Cambridge Isotope Laboratories Inc., Andover, MA, USA). A 2 ml amber glass vial was placed in the whorl of the maize plant inside the chamber. Each vial contained 20 mg of either labeled or unlabeled indole; empty vials were used for controls. Each vial was sealed with a PTFE screw cap with a rubber septum pierced three times with pinprick holes to allow a slow release of volatile indole (c. 400 ng h^{-1}). Plants were maintained in the volatile collection chambers for 48 h, at which point they were removed and the whorl tissue collected, and flash frozen in liquid nitrogen for analyses.

Pathway of Origin Determination in Untargeted Metabolomics analysis

Flash-frozen tissue from maize inbred B73 plants exposed to unlabeled volatile indole or heavy isotope-labeled volatile indole ($^{13}C_8$;^{15}N) were extracted in 70% methanol. Metabolite extracts were analyzed by untargeted liquid chromatography-mass spectrometry (LC-MS) in negative and positive ionization modes following the instrument settings described in Simpson et al. (2021). The LC-MS raw data (.D files) were converted to mzXML format by MSconvert software for analysis by an XCMS-based R pipeline, Pathway of Origin Determination in Untargeted Metabolomics (PODIUM), to identify metabolite features derived from indole. The initial peak detection was performed using the centWave algorithm by following parameters: ppm = 30, peakwidth = c(3, 27), snthresh = 10, prefilter = c(3, 100), integrate = 1, nslaves = 10. Detected peaks were grouped by minfrac = 0.33, minsamp = 1, mzwid = 0.025, bw = 10, and max = 50. Retention time correction and peak alignment were performed by the obiwarp method with response = 100. After alignment, peaks were grouped again, and groups eluted before 90 s were removed. The fillPeaks function was used to fill peaks missing in the initial peak detection. From the detected peaks, PODIUM identified co-eluting peak pairs with the number of expected labeled atoms nLable = 9 and the mass difference between the labeled and unlabeled atom mLabel = 1.00265227, which was calculated by [8 9 (^{13}C–^{12}C) + (^{15}N–^{14}N)]/9. A PODIUM-post criterion was applied to improve the confidence of the identification of peak pairs. This criterion stipulated that in unlabeled indole-fed samples, unlabeled signals should be twofold or greater than labeled signals.

Benzoxazinoid analysis by targeted LC-MS/MS

Benzoxazinoid content was measured by liquid chromatography-tandem triple quadrupole mass spectrometry (LC-MS/MS). Flash frozen tissue from plants exposed to unlabeled volatile indole or volatile heavy isotope-labeled indole [$^{13}C_8$,^{15}N] were extracted in (98 : 2 (v/v)) methanol: acetic acid (200 mg in 1 ml) containing 5 1g ml^{-1} 2-benzoxazolinone (2-BOA) (Sigma-Aldrich) as an internal standard. Samples were sonicated for 20 min, centrifuged for 10 min, and the supernatant was filtered through a 2-micron nylon filter. Extracted samples were run on a Thermo Scientific TQ Quantis MS equipped with a Thermos Vanquish LC. Compounds were separated on an Agilent Eclipse Plus C18 RRHD column (50 9 2.1 mm, 1.8 1m particle size) (Agilent, Santa Clara, CA, USA) held at 30°C using solvent A (0.1% (v/v) formic acid in water) and solvent B (0.1% (v/v) formic acid in acetonitrile) at a flow rate of 0.4 ml min^{-1}. The separation gradient was held at 5% B for 2 min then increased to 100% B over 20 min, held at 100% B for 2 min, then a returned to 5% B over 7 min. MS parameters were ion transfer tube temperature 300°C, vaporizer temperature 350°C, arbitrary gas units: sheath 50, aux 15, sweep 2 and collision energy of 20 V. Single reaction monitoring (SRM) of $[M + H]^+$ and $[M - H]^-$ ions were used in separate negative (−2500 V) and positive (+3500 V) ion methods to quantify labeled and unlabeled benzoxazinoids using the following transitions and retention times (RT) in negative ionization mode: DIMBOA-Glc (372.09 to 192.03 m/z; RT: 4.76 min), and labeled [$^{13}C_8$,^{15}N]DIMBOA-Glc (381.09 to 201.03 m/z; RT 4.76 min); DIM_2BOA-Glc (402.01 to 194.05 m/z, RT: 5.01 min), and labeled [$^{13}C_8$,^{15}N]DIM_2BOA-Glc (411.10 to 203.05 m/z, RT:5.01 min). In positive ionization mode HDMBOA-Glc (388.12 to 194.05 m/z, RT: 6.06 min), and labeled [$^{13}C_8$,^{15}N]HDMBOA-Glc (397.12 to 235.07 m/z, RT: 6.06 min); DIMBOA (212.06 to 166.05 m/z, RT:8.54); labeled [$^{13}C_8$,^{15}N]DIMBOA (221.06 to 174.05 m/z, RT:8.54); and 2-BOA internal standard (136.0 to 80.0 m/z, RT:5.51 min).

Tryptophan analysis by LC-MS/MS

Flash-frozen tissue from plants exposed to volatile heavy isotope-labeled indole [$^{13}C_8$,^{15}N] or nontreated control plants were extracted in methanol (200 mg in 1 ml), centrifuged for 10 min, and the supernatant filtered through a 2-micron nylon filter. Extracted samples were analyzed with the described LC-MS/MS, mobile phases, and flow rate for benzoxazinoid analysis above. A Thermo Hypersil Gold (150 × 2.1 mm, 1.9 µm particle size) C18 HPLC column was used at 30°C with a gradient as follows: 0% B held for 1 min, increased to 20% B over 4 min, increased to 100% B over an additional 4 min, held at 100% B for 2 min, returned to 0% B over 2 min, and held at 0% B for 5 min. MS parameters were +4000 V, ion transfer tube temperature 300°C, vaporizer temperature 350°C, arbitrary gas units: sheath 50, aux 5, and sweep 1. SRM was used to quantify labeled and unlabeled tryptophan in positive ionization mode. Unlabeled tryptophan ($[M + H]^+$ precursor 205.16 m/z) was monitored with a quantification mass transition to 188.21 m/z and qualification transition to 146.13 m/z. Isotope-labeled [$^{13}C_8$,^{15}N] tryptophan ($[M + H]^+$ precursor 214.16 m/z) was monitored with a quantification transition to 197.21 m/z and qualification transition to 155.13 m/z. The collision energy for the quantification and qualifications SRMs were 17.43 V and 10.01 V, respectively.

IAA analysis by LC-MS/MS

IAA concentration was determined by LC-MS/MS as in Uyehara et al. (2023). Briefly, 100 mg of frozen ground tissue was extracted in dichloromethane with addition of a d5-IAA internal standard. Extracted samples were evaporated under nitrogen, resuspended in methanol and filtered through a 2-micron nylon filter. Extracted samples were analyzed with the LC-MS/MS and column described for benzoxazinoid analysis. The mobile phase was solvent A (0.2% (v/v) acetic acid in water) and solvent B (0.2% (v/v) acetic acid in acetonitrile) with a 0.45 ml min^{-1} flow rate and column temperature at 25°C, using a 30 min gradient (held at 20% B for the first 3 min, increased to 100% B over 17 min, then held at 100% B for 5 min, returning to 20% B over 5 min, and finished as a hold for 5 min at 20% B). MS parameters were −3000 V, ion transfer tube temperature 300°C, vaporizer temperature 350°C, arbitrary gas units: sheath 50, aux 15, sweep 2 and collision energy of 10 V. SRM was used to quantify labeled and unlabeled IAA using the following ion pairs and retention times in negative ionization mode: IAA (174 to 130 m/z, RT 4.75 min), d5-IAA (179 to 135 m/z, 4.71 min); [$^{13}C_8$: ^{15}N] IAA (183 to 139 m/z, 4.75 min).

Indole priming assays and green leaf volatile quantification by GC-MS

To induce priming plants were placed in volatile collection chambers (one pot containing two 3-wk-old plants per chamber) with or without volatile indole exposure for 20 h. GLV production was then induced by wounding a 4 cm^2 section of the fourth leaf of each plant with a razor blade and painting 5 µl of boiled Spodoptera frugiperda (J.E. Smith) (Lepidoptera, Noctuidae) regurgitant on the wound site. Chambers were closed immediately after treatment and headspace volatiles were collected on columns containing SuperQ resin for 30 min with an incoming airflow of 1.7 l min^{-1} and a vacuum through the SuperQ columns of 0.7 l min^{-1}. After collection, the columns were removed from the chambers and captured volatiles were eluted from the columns in 150 µl of methylene chloride. The above-ground fresh weight of the plant tissue in each chamber was recorded. Volatile compounds were analyzed and quantified on an Agilent 7890A gas chromatograph with a DB5-MS column (30 m × 0.250 mm × 0.25 µm) coupled to an Agilent 7000 triple quadrupole mass spectrometer. Two microliters of eluted volatiles were injected onto the GC using a splitless injection and separated with a flow of 0.94 ml min^{-1} and a temperature gradient of 30°C for 2 min followed by a temperature ramp at 10°C min^{-1} to 250°C, and a hold for 1 min at 250°C. Mass spectrometry was performed using an electron ionization source at 220°C operated in MS1 scan mode from m/z 45–550 with a 250 ms scan time and steps of m/z 0.1. The identities of GLVs quantified using peak area of the total ion chromatograph were confirmed using authentic standards as (3Z)-hexenal (RT 6.30 min), (3Z)-hexenol (RT 7.25 min), and (3Z)-hexenyl acetate (RT 9.82 min).

Results

Labeled volatile indole is converted into the benzoxazinoid DIMBOA-glucoside in maize

To investigate if volatile indole was taken up by the leaves of maize seedlings and converted into other indole-derived metabolites, maize seedlings were exposed to heavy isotopically labeled or unlabeled volatile indole for 48 h, and extracts were analyzed using untargeted LC-MS. Pathway of Origin Determination in Untargeted Metabolomics (Simpson et al., 2021) was used to identify six metabolite features generated from volatile indole. To identify metabolites derived from indole through direct metabolism, metabolite features that incorporate the intact indole structure, leading to an increase of nine Daltons in their mass-to-charge (m/z) values, were specifically investigated. Comparing the metabolic profiles of maize tissues treated with unlabeled volatile indole and [$^{13}C_8$,^{15}N]-indole, six metabolite features were identified as volatile indole derivatives (Table 1). To investigate the identity of these metabolite features, m/z values were

Table 1 PODIUM-identified volatile indole-derived metabolite features in maize leaves.

Feature ID	m/z	Retention time (s)	Ionization mode
M467T384	467.1662	383.63	Positive
M165T502	165.0455	502.08	Positive
M369T1375	369.2971	1374.63	Positive
M785T1389	784.5388	1389.09	Positive
M372T502	372.0942	502.32	Negative
M745T502	745.1956	502.12	Negative

searched against the METLIN database. Peak M371T502 was assigned as a DIMBOA-glucoside. Peak M165T502 may correspond to the neutral mass for the benzoxazinoid breakdown product 6-methoxy-2-benzoxazolinone (MBOA). These observations suggest that exogenous volatile indole may be metabolized into benzoxazinoids in maize.

To test if volatile indole was converted into DIMBOA-glucoside in plants, targeted LC-MS/MS analysis was performed to measure benzoxazinoid accumulation in wild-type (bzW22) maize plants that were exposed to heavy isotope-labeled [$^{13}C_8$,^{15}N] volatile indole for 48 h. Plants exposed to labeled volatile indole had equivalent levels of nonlabeled DIMBOA-glucoside to nontreated plants (Fig. 2a,b), and they accumulated [$^{13}C_8$,^{15}N] ring-labeled DIMBOA-glucoside (Fig. 2d) that was not present in the nontreated control (Fig. 2c). The amount of labeled DIMBOA-glucoside measured was equivalent to 0.3% of the total DIMBOA-glucoside in the tissues. These data confirm that plants can indeed uptake volatile indole and convert it to benzoxazinoids such as DIMBOA-glucoside. Plants that were exposed to [$^{13}C_8$,^{15}N] volatile indole were examined for the presence of [$^{13}C_8$,^{15}N]-labeled isotopes in other benzoxazinoids, including DIM_2BOA-glucoside and HDMBOA-glucoside, but no detectable accumulation of heavy isotopes in these compounds was observed at the time points tested. These data indicate that, of the benzoxazinoids examined, labeled indole is primarily converted into DIMBOA-glucoside.

Conversion of volatile indole to DIMBOA-glucoside occurs via the endogenous benzoxazinoid biosynthesis pathway

In maize, BX1 converts indole-3-glycerolphosphate to indole as the first step of the benzoxazinoid biosynthesis pathway (Frey et al., 1997; Melanson et al., 1997; Fig. 1). BX1-produced indole is then converted into indolin-2-one by BX2. The conversion of volatile indole into DIMBOA-glucoside may therefore be due to it being a substrate for BX2 and thus entering the benzoxazinoid biosynthesis pathway. To test this hypothesis, bx2 loss-of-function mutant plants and their corresponding wild-type control (bzW22) were exposed to heavy isotope-labeled [$^{13}C_8$,^{15}N] volatile indole and the accumulation of heavy isotope [$^{13}C_8$,^{15}N] DIMBOA-glucoside was measured. Exposure to labeled volatile indole led to the accumulation of labeled DIMBOA-glucoside in wild-type plants but no detectible accumulation of labeled DIMBOA-glucoside was observed in bx2 mutant plants (Fig. 3a). These data show that a functional BX2 enzyme is required to convert volatile indole into benzoxazinoids.

To determine if BX2 was the exclusive point of entry of volatile indole to the benzoxazinoid biosynthesis pathway, bx1 loss of function mutant plants and their corresponding wild-type plants were exposed to heavy isotope-labeled [$^{13}C_8$,^{15}N] volatile indole. Accumulation of heavy isotope-labeled [$^{13}C_8$,^{15}N] DIMBOA-glucoside was observed in both bx1 mutant and wild-type plants exposed to labeled volatile indole (Fig. 3b). These data show that, unlike BX2, a functional BX1 enzyme is not required for the conversion of volatile indole into benzoxazinoids.

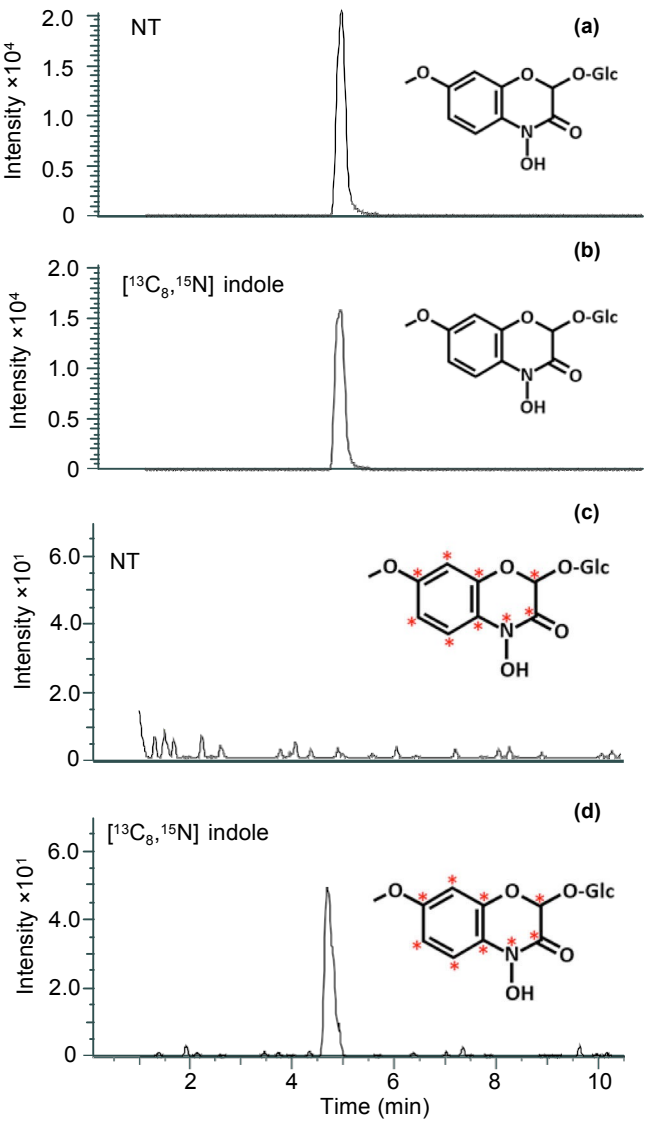

Fig. 2 Conversion of volatile-labeled indole into DIMBOA-glucoside occurs in wild-type maize plants. DIMBOA-glucoside (2,4-dihydroxy-7-methoxy-2H-1,4-benzoxazin-3(4H)-one-b-D-glucopyranose)was measured by SRM at 372.09 to 192.03 m/z in nontreated (NT) plants (a) and plants exposed to volatile-labeled [$^{13}C_8$,^{15}N] indole (b). Incorporation of indole into DIMBOA-glucoside was monitored by measuring [$^{13}C_8$,^{15}N] ring-labeled DIMBOA-glucoside by SRM at 381.09 to 201.03 m/z. No peak for [$^{13}C_8$,^{15}N] DIMBOA-glucoside was observed in NT plants (c), while a peak for labeled DIMBOA-glucoside was observed in plants exposed to volatile-labeled [$^{13}C_8$,^{15}N] indole (d). Panels show representative chromatograms and structures of the compounds monitored. (*) show the positions of heavy isotope atoms.

To confirm that the bx2 and bx1 mutants were deficient in DIMBOA-glucoside biosynthesis, unlabeled DIMBOA-glucoside levels were quantified in both mutant lines and their corresponding wild-types. No detectable amounts of unlabeled DIMBOA-glucoside were observed in either the bx2 mutants (Fig. 3c) or the bx1 mutants (Fig. 3d) irrespective of their exposure to labeled volatile indole, while DIMBOA-glucoside accumulation was readily detectable in the corresponding wild-type

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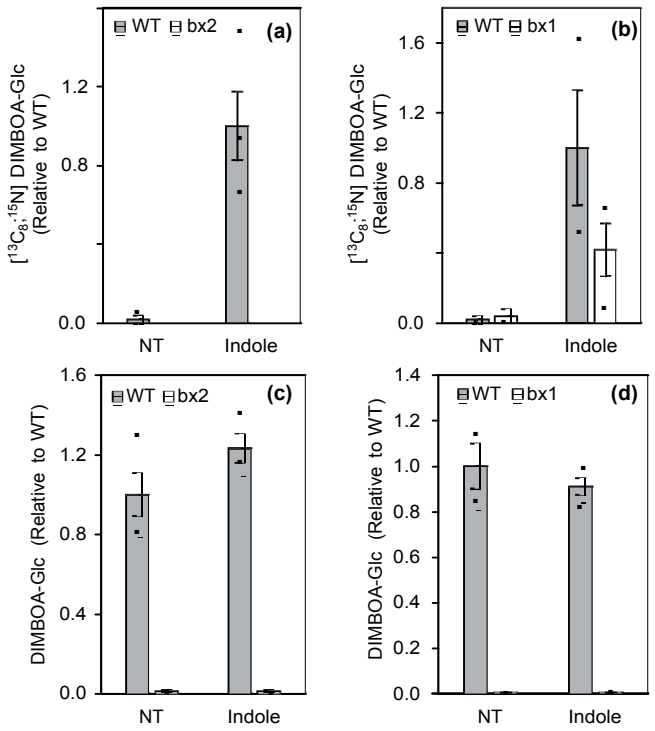

Fig. 3 Conversion of volatile-labeled indole into DIMBOA-glucoside occurs in the bx1 mutant but not the bx2 mutant of maize. Mutants in the benzoxazinoid biosynthesis genes bx2 (indole-2-monooxygenase) and bx1 (indole-3-glycerolphosphate lyase) and their corresponding wild-types (WTs) were either not treated (NT) or exposed to heavy isotope volatile [$^{13}C_8$,^{15}N] indole for 48 h. The relative accumulation of heavy isotope [$^{13}C_8$,^{15}N] DIMBOA-glucoside (2,4-dihydroxy-7-methoxy-2H-1,4-benzoxazin-3(4H)-one-b-D-glucopyranose) in bx2 (a) and bx1 (b) mutants and their respective WT plants was determined by LC-MS–MS. Relative abundance of total unlabeled DIMBOA-glucoside was also assessed in bx2 (c) and bx1 (d) mutant and WT plants. Graphs show mean of n = 4 with SEM error bars.

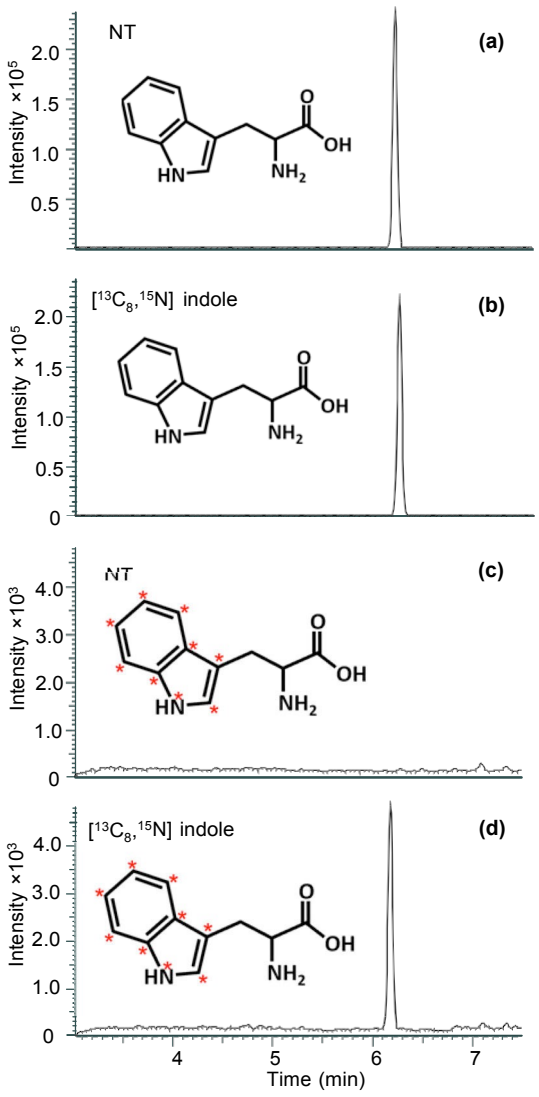

Fig. 4 Conversion of volatile-labeled indole into tryptophan occurs in wild-type maize. Tryptophan was measured by SRM (single reaction monitoring) at 205.162 to 188.208 m/z in nontreated (NT) plants (a) and plants exposed to volatile-labeled [$^{13}C_8$,^{15}N] indole (b). Incorporation of indole into tryptophan was monitored by measuring [$^{13}C_8$,^{15}N] ring-labeled tryptophan by SRM 214.162 to 197.208 m/z. No peak for [$^{13}C_8$,^{15}N] tryptophan was observed in NT plants (c), while a peak for labeled tryptophan was observed in plants exposed to volatile-labeled [$^{13}C_8$,^{15}N] indole (d). Panels show representative chromatograms and structures of the compounds monitored. (*) show the positions of heavy isotope atoms.

plants. These data indicate that volatile indole enters the benzoxazinoid pathway at the level of BX2 and is converted into DIMBOA-glucoside by the endogenous benzoxazinoid enzymes.

Volatile indole is also converted into tryptophan in maize

Benzoxazinoid biosynthesis is not the only metabolic pathway in maize in which indole serves as an intermediate. It is also an intermediate in the synthesis of the amino acid tryptophan, where the indole-3-glycerolphosphate lyase (TSA1 – tryptophan synthase alpha subunit1) converts indole-3-glycerolphosphate into indole. This protein forms a complex with the tryptophan synthase beta (TSB) subunits (ORP1 – orange pericarp1 and ORP2 – orange pericarp2) which convert indole to tryptophan (Wright et al., 1992; Kriechbaumer et al., 2008).

To assess whether volatile indole is converted into tryptophan by maize leaves, wild-type (bzW22) maize plants were exposed to heavy isotope-labeled [$^{13}C_8$,^{15}N] volatile indole for 48 h and the accumulation of both unlabeled and labeled [$^{13}C_8$,^{15}N] tryptophan monitored using LC-MS–MS. The amount of unlabeled tryptophan in nontreated (NT) plants (Fig. 4a) was equivalent to that in plants exposed to labeled [$^{13}C_8$,^{15}N] volatile indole (Fig. 4b). As expected, no heavy isotope-labeled tryptophan was observed in plants that were not exposed to labeled indole (Fig. 4c). In contrast, plants that were exposed to labeled indole for 48 h accumulated labeled [$^{13}C_8$,^{15}N] tryptophan (Fig. 4d) that accounted for c. 0.4% of total tryptophan in the leaf tissues. These data demonstrate that volatile indole can be converted into tryptophan by maize plants.

To assess whether the benzoxazinoid biosynthesis pathway and tryptophan biosynthesis pathways compete for volatile indole, the accumulation of [$^{13}C_8$,^{15}N] tryptophan was first assessed in bx1 mutant plants, and their corresponding wild-type exposed for 48 h to labeled volatile indole or no-treatment controls (Fig. 5a). Equivalent amounts of labeled tryptophan accumulated in the bx1 plants and their corresponding wild-type when exposed to labeled volatile indole. The bx2 mutants and their corresponding wild-type plants also accumulated equivalent amounts of labeled tryptophan when exposed to labeled volatile indole (Fig. 5b). Both bx1 mutant plants (Fig. 5c), and the bx2 mutants (Fig. 5d) contained equivalent amounts of unlabeled tryptophan as their corresponding wild-type plants. These data indicate that the benzoxazinoid and tryptophan biosynthesis pathways in maize do not compete for volatile indole.

Indole is also an intermediate in the biosynthesis of the plant hormone IAA in the tryptophan-dependent and possibly in the tryptophan-independent pathways (Wang et al., 2015). To investigate if volatile indole was incorporated into IAA, wild-type plants were exposed to labeled volatile indole and the accumulation of [$^{13}C_8$,^{15}N] IAA was monitored using LC-MS/MS. No detectible accumulation of labeled IAA was observed in plants exposed to labeled volatile indole (Supporting Information Fig. S1), indicating that volatile indole is not a prominent source of substrate for auxin biosynthesis.

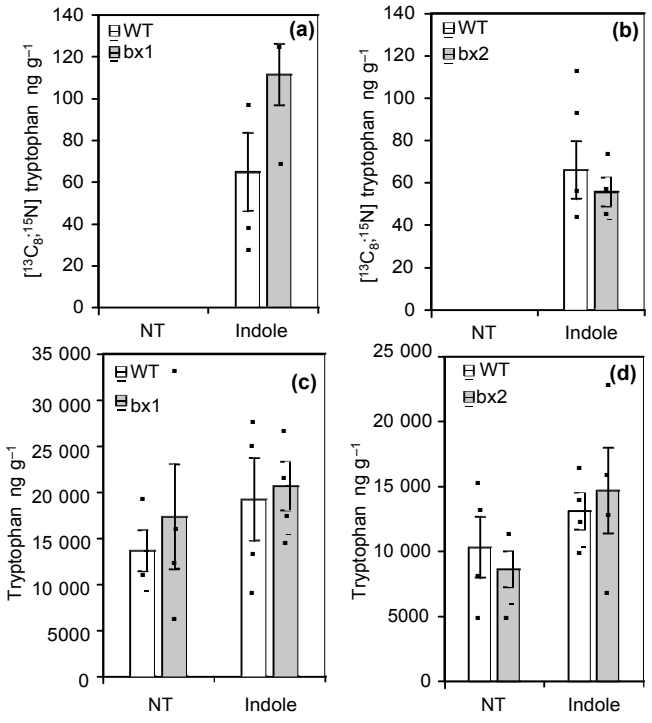

Fig. 5 Conversion of volatile-labeled indole into tryptophan occurs in both the bx1 and bx2 mutants of maize. Mutants in the benzoxazinoid biosynthesis genes and bx1 (indole-3-glycerolphosphate lyase) and bx2 (indole-2-monooxygenase) their corresponding wild-types (WTs) were either not treated (NT) or exposed to heavy isotope volatile [$^{13}C_8$,^{15}N] indole for 48 h. The accumulation of heavy isotope [$^{13}C_8$,^{15}N] tryptophan in bx1 (a) and bx2 (b) mutants and their respective WT plants was determined by LC-MS–MS. Amount of total unlabeled tryptophan was also assessed in bx1 (c) and bx2 (d) mutant and WT plants. Graphs show mean of n = 4 with SEM error bars. No significant differences were observed between mutant and wild type plants.

The bx2 mutant plants showed elevated GLV production in response to wounding and S. frugiperda regurgitant irrespective of indole exposure

Exposure to volatile indole has been shown to induce priming in maize that results in increased production of herbivore-induced volatiles such as GLVs after wounding and herbivore regurgitant treatment (Erb et al., 2015). To determine if indole-mediated priming of GLVs is dependent upon the conversion of indole into benzoxazinoids, the ability of exogenous indole to prime herbivore-induced GLVs in the bx2 mutant was assessed (Fig. 6). Exposure of wild-type (bzW22) plants to volatile indole led to a significant increase in production of the GLVs (3Z)-hexenal (Fig. 6a), (3Z)-hexenol (Fig. 6b), and (3Z)-hexenyl acetate (Fig. 6c) in response to wounding and treatment with S. frugiperda regurgitant compared to plants without indole exposure. In contrast, bx2 plants exposed to volatile indole had no significant difference in induced GLV production compared to bx2 plants without indole exposure. However, the total amount of GLVs produced in bx2 plants without indole exposure was equivalent to that in wild-type plants with indole exposure, and significantly higher than in wild-type plants not-exposed to indole. These data indicate that indole-induced priming of GLV induction is not dependent on benzoxazinoid production. Furthermore, the increased GLV response of bx2 mutants to wounding and treatment with S. frugiperda regurgitant may be due to the block in benzoxazinoid formation from endogenous indole leading to increased free indole production in these lines.

Discussion

In this study, we show that herbivore-induced volatile indole can be taken up into the leaves of maize plants and converted into the defense compound DIMBOA-glucoside (Fig. 2) and that this activity depends on the benzoxazinoid biosynthesis gene BX2 (indole-2-monooxygenase) (Fig. 3). No incorporation of indole into the benzoxazinoids DIM_2BOA-glucoside, and HDMBOA-glucoside was observed in our experiments, perhaps reflecting the positions of these metabolites further downstream in the benzoxazinoid biosynthesis pathway (Fig. 1), or the higher abundance of DIMBOA-glucoside compared to other benzoxazinoids in the leaves of young maize plants (Cambier et al., 2000).

Benzoxazinoids are important defense compounds produced by several plant families, including agriculturally important members of Poaceae such as maize and wheat (Triticum aestivum). These metabolites are phytoanticipins and are stored in their less toxic glucoside form in the plant vacuole. Disruption of cell integrity caused by herbivory releases the glucosides from the vacuole and exposes them to a plastid localized b-glucosidase converting them to toxic aglycones (e.g. DIMBOA-glucoside to DIMBOA). The aglycones' toxicity is due to reactivity with NH_2

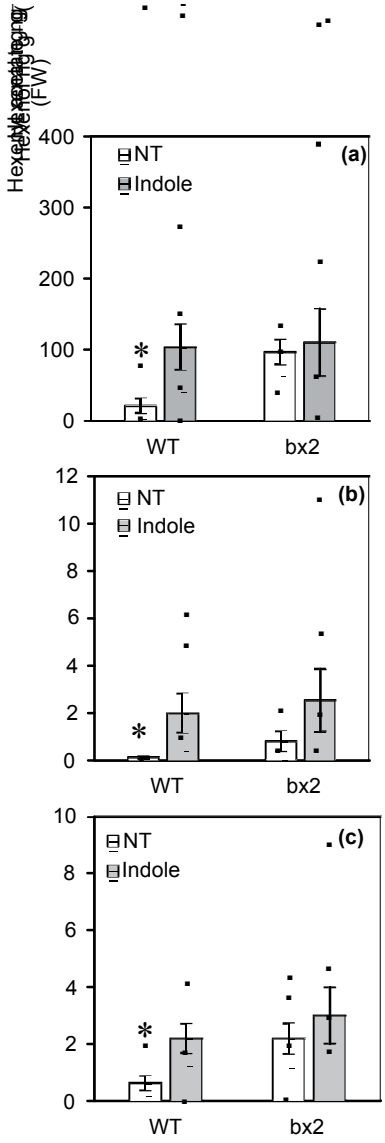

Fig. 6 Indole priming of green leaf volatiles in maize does not depend on benzoxazinoids. bx2 (indole-2-monooxygenase) mutant plants and their corresponding wild-type (WTs) were either not treated (NT) or exposed to volatile indole for 20 h. Leaves were then wounded and treated with Spodoptera frugiperda regurgitant. Headspace volatiles were collected for 30 min after wounding and the levels of the green leaf volatiles (3Z)-hexenal (a), (3Z)-hexenol (b) and (3Z)-hexenyl acetate (c) were measured by GC-MS. Graphs show mean of n = 8 with SEM error bars. Treatment with a significant difference by Student's t-test $P < 0.05$ are indicated by *.

and SH nucleophilic groups of proteins and other biomolecules in the attacking herbivore (Frey et al., 2009). Our observation that volatile indole can be taken up by the leaves of maize plants and converted into DIMBOA-glucoside indicates that indole may act to prime defenses in neighboring maize plants or in distal tissues of the same plant by directly contributing to the accumulation of benzoxazinoid phytoanticipins.

The amount of indole released by maize can vary greatly depending on the variety of maize and the inducing stress. Headspace analysis of a range of maize inbreds infested with Spodoptera littoralis showed indole emission rates between 0 and 4 **1**g h^{-1} g^{-1} (dry weight) (Degan et al., 2004), while wounding and larval oral secretion applications transiently induced indole emission at 50 ng h^{-1} in the maize hybrid Delprim (Erb et al., 2015), and 20 ng h^{-1} g^{-1} (fresh weight) in maize inbred B73 (Block et al., 2018) respectively. In our study, exposure of young maize plants to labeled volatile indole at 400 ng h^{-1} resulted in incorporation of the heavy isotope label into 0.3% of the total pool of DIMBOA-glucoside. In these unstressed plants, the relatively small addition to the DIMBOA-glucoside pool would not necessarily impact the resistance to pests, however, it remains an open question as to if this small effect is due to a limitation of the substrate (indole) or to other restrictions on the flux through the benzoxazinoid pathway. The particularly high levels of benzoxazinoids in young maize seedlings (0.1–0.3% fresh weight) (Meihls et al., 2013) may also act to mask the contribution of volatile indole to DIMBOA-glucoside production in these tissues. Due to the differences in endogenous benzoxazinoid profiles of maize during development (Cambier et al., 2000), one can hypothesize that the relative contribution of volatile indole to the benzoxazinoid pool may change depending on the age and tissue of the plant.

The production of certain benzoxazinoids is induced in response to herbivory and other biotic stresses (Oikawa et al., 2004; Glauser et al., 2011; Huffaker et al., 2011). It is therefore possible that volatile indole could act as a supplemental substrate pool when the benzoxazinoid pathway is induced, and thus enhance the induction of benzoxazinoid synthesis. However, whether the amount of volatile indole produced can contribute to benzoxazinoid levels (in neighboring plants or in distal or adjacent tissues of the same plant) to the extent that herbivore growth is impacted, remains an open question.

In addition to its toxicity, DIMBOA also functions as an extracellular signal for chitosan-induced callose deposition (Ahmad et al., 2011). This potential role for benzoxazinoids in defense signaling led us to test if the conversion of volatile indole to benzoxazinoids was involved in indole-mediated GLV priming in maize. Our data showed that, in line with previous studies (Erb et al., 2015), exposure to volatile indole led to enhanced GLV production following wounding and treatment with S. frugiperda regurgitant in wild-type maize plants (Fig. 6). In contrast, the bx2 mutant plants that are incapable of converting indole to benzoxazinoids did not show significantly different GLV induction in plants that were exposed to indole compared to those that had no prior indole exposure. These data suggest that bx2 plants are not being primed by exogenous indole. However, the GLV production in the bx2 mutant plants, even without indole exposure, was equivalent to that in the wild-type with indole exposure, and significantly higher than that in wild-type plants without indole exposure. The most likely explanation for this phenotype is that the bx2 plants that block indole conversion to benzoxazinoids accumulate higher levels of endogenous indole than the wild-type plants, leading to a constitutive priming phenotype, that exogenous indole cannot further enhance. These data indicate that indole priming of GLV induction is not dependent on benzoxazinoids.

In addition to the incorporation of volatile indole into benzoxazinoids, we also observed its incorporation into tryptophan (Fig. 4). This was surprising because the a- and b-subunits of the

tryptophan synthase bi-enzyme complex form an interconnecting channel that transfers the indole intermediate between the two subunits, preventing the escape of indole during tryptophan synthesis (Dunn, 2012). A priori, one would expect such metabolic channeling to limit the incorporation of 'free' indole into tryptophan, as much as it limits the release of channeled indole into the cell. These data show that exogenous indole is converted into tryptophan at a rate of c. 0.4% of total tryptophan after 48 h exposure. This relatively low incorporation probably reduces the likelihood of exogenous indole significantly impacting the accumulation of the plethora of tryptophan-derived metabolites. In line with this, no significant incorporation of free indole into IAA was observed under the conditions tested (Fig. S1). The ability of volatile indole to enter both the tryptophan and benzoxazinoid biosynthesis pathways despite the apparent channeling of indole in tryptophan biosynthesis, as seen by the trace levels of DIMBOA-glucoside in the bx1 mutants, suggests that the pathways are more able to accept indole than release it.

Our heavy isotope volatile indole labeling experiments suggest that the role of indole in distal defense via conversion into other defense/signaling compounds may in part lie in its conversion to benzoxazinoids. However, this does not encompass all the roles for indole in priming, as priming of GLV induction by indole is not dependent on intact benzoxazinoid biosynthesis pathways. It is likely, then, that indole acts directly in these processes. Indole has been shown to regulate membrane potentials. For instance, indole blocks E. coli cell division by acting as a proton ionophore and reducing the electrochemical potential across the cytoplasmic membrane (Chimerel et al., 2012). With this capacity in mind, it is possible that indole could be priming the plant cells by altering membrane potentials. Another possibility is that indole is a ligand for a plant receptor protein. While no indole receptors have so far been described in plants, indole and indole derivatives are thought to be ligands of the aryl hydrocarbon receptor (AhR), a cytosolic ligand-activated transcription factor in animal cells (Vyhlídalová et al., 2020). The ligand plasticity in animal AhR mirrors that seen in plant auxin receptors (Uzunova et al., 2016), and this plasticity coupled with the structural similarity of indole and auxins raises the intriguing possibility that some of the array of auxin receptors (Pérez-Henríquez & Yang, 2023) may also bind indole in an agonistic or antagonistic fashion. The auxin IAA is suppressed in maize in response to herbivory by Helicoverpa zea (corn earworm) (Schmelz et al., 2003), suggesting that alterations in auxin-adjacent signaling may impact induced responses to herbivory in maize. Though much remains unknown as to how indole, and other volatiles, function as interplant signals, their importance in the communal nature of plant defense is an exciting area of current and future research.

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Competing interests

None declared.

Author contributions

Project was conceived by AKB; experiments were designed and conducted by AS, Z-WL, Q-BL, KR, CH, CR, and AKB; Data were analyzed and interpreted by AS, Z-WL, GJB, JK, CC, and AKB; supervision and guidance were provided by GJB, JK, CH, CC, CR and AKB; paper was written by AKB with input from all authors.

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Data availability

Data is available in Table S1.

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Supporting Information

Additional Supporting Information may be found online in the Supporting Information section at the end of the article.

Fig. S1 Analysis of indole-3-acetic acid (IAA) in wild-type maize plants exposed to volatile heavy isotope indole showed no detectable accumulation of labeled IAA.

Table S1 Liquid chromatography-mass spectrometry data file.

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