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Systemic defense priming by *Pseudomonas putida* KT2440 in maize depends on benzoxazinoid exudation from the roots

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Exudation of benzoxazinoid metabolites from roots of young maize seedlings recruits the rhizobacterial strain *Pseudomonas putida* KT2440 from the soil to the rhizosphere. In this study, we have investigated whether these rhizobacteria are beneficial for maize by eliciting systemic defense priming. Root colonization of the maize hybrid cultivar Delprim by *P. putida* primed wound- and jasmonic acid (JA)-inducible emission of aromatic and terpenoid volatiles, but not the emission of the green leaf volatile (*Z*)-3-hexenyl acetate. Furthermore, root colonization by *P. putida* primed stress-inducible transcription of the JA-dependent gene *SerPIN*, whereas JA-dependent induction of the *MPI* gene was unaffected. Systemic priming of *SerPIN* by *P. putida* only occurred in benzoxazinoid-producing plants, and was absent in benzoxazinoid-deficient plants. The results from this study suggest that root colonization by *P. putida* primes a selection of JA-dependent defenses in Maize, which is reliant on benzoxazinoid exudation from the roots.

The relationship between cereal plants and soil bacteria has evolved over 60 million years, the time at which the first firm evidence for grass pollens exists.[1] This long period of co-evolution has resulted in a wide-range of cereal-biotic interactions, ranging from beneficial to pathogenic. Selected beneficial strains of non-pathogenic soil bacteria can promote plant growth. This interaction has attracted a great deal of interest as it provides opportunities for exploitation in sustainable food production by cereals. *Pseudomonas* spp., particularly *P. fluorescens*[2] and *P. putida*,[3,4] have been extensively studied for their ability to promote plant growth. Plant growth-promotion by rhizobacteria has been ascribed to various mechanisms, including nitrogen fixation, solubilization of essential plant nutrients, production of plant-like growth hormones, inhibition of growth-repressing ethylene production and direct antagonism of growth-suppressing plant pathogens in the rhizosphere.[5] Recently, ref. 6 proposed an alternative mechanism: rhizobacteria induce growth promotion in Arabidopsis by inducing a starvation-like response. The authors proposed that the resulting increase in soluble carbohydrates in the plant not only benefits bacteria on the rhizoplane, but may also contribute to growth promotion.

In addition to the mechanisms noted above, some growth-promoting bacteria are capable of improving plant health via eliciting an induced systemic resistance (ISR) response. In this case, colonization by rhizobacteria results in long-lasting resistance against a broad range of pathogens.[7] The plant signaling mechanisms mediating ISR have been studied extensively in Arabidopsis following root colonization by *P. fluorescens* WCS417r.[8,9] In this model system, ISR is based on systemic priming of the plant immune system, resulting in a quicker and more potent accumulation of ethylene- and JA-dependent gene transcripts and callose-rich papillae after pathogen attack.[10-12] *Pseudomonas*-elicited ISR has also been reported in a variety of crop-pathogen partnerships, including Cotton-*Fusarium oxysporum*,[13] Cucumber-*Colletotrichum orbiculare*[14] and Rice-*Magnoporthe oryzae*.[15] In the latter, ISR is associated with an augmented capacity for pathogen-induced callose deposition and functional responsiveness to the plant hormone JA.

We recently reported attraction of the soil bacterium *P. putida* KT2440 cells to the Maize rhizoplane in response to exudation of the benzoxazinoid metabolite 2,4-dihydroxy-7-methoxy-2*H*-1,4-benzoxazin-3(4*H*)-one (DIMBOA).[16] DIMBOA is exuded in relatively high concentrations from the roots of young Maize plants,[16] and is known for its insecticidal and phytotoxic activities.[17,18] In addition, DIMBOA plays a signaling role in aboveground defenses against aphids and fungi, where it functions as an apoplastic signal for induction of cell wall defense.[19] Despite these defense activities, roots of benzoxazinoid-producing Maize lines are subject to higher levels of *P. putida* KT2440 root colonization than benzoxazinoid-deficient Maize lines carrying a mutation in the *ZmBX1* gene. In vitro experiments revealed that this difference is based on enhanced tolerance of *P. putida* KT2440 to high concentrations of DIMBOA, combined with a positive chemotactic response to the compound. However, it remained untested
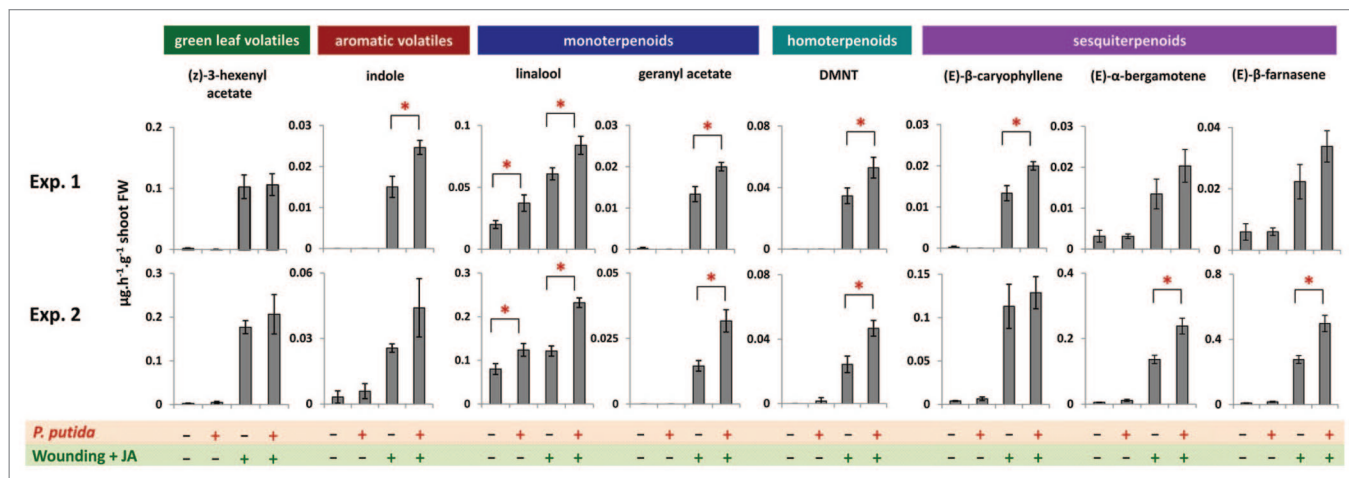
Figure 1. Emission of volatile organic compounds (VOCs) from maize leaves upon root-colonization by *Psedomonas putida* KT2440 and subsequent defense elicitation by leaf wounding and JA application. Shown are average emission rates (± SEM) from two independent experiments over a 24 h collection period. Asterisks indicate statistically significant differences between bacterized and non-bacterized control groups (Student's t-test; $p < 0.05$).

whether Maize plants benefit from root colonization by *P. putida* and to what extent such beneficial host effects rely on benzoxazinoid-dependent recruitment of bacteria. In this study, we have investigated whether *P. putida* bacteria prime JA-dependent defense mechanisms in Maize, and whether these responses rely on the host plant's ability to produce benzoxazinoids.

Results and Discussion

Analysis of VOC emissions from intact Maize plants indicated that root colonization by *P. putida* has no direct effects on emission of the majority of volatiles tested (**Fig. 1**). The only volatile showing increased emission rates in *P. putida*-colonized plants was the monoterpene (±)-Linalool. Linalool has been shown to affect insect behavior.[26-28] Ref. 29 reported that VOC blends with higher levels of (-)-isomer deterred oviposition on *Datura wrightii* by *Manduca sexta* moths. More recently, ref. 30 reported contradictory data, and showed that increased emission of (+)-Linalool from transgenic tobacco plants deters oviposition by the moth *Helicoverpa armigera*, but has no effect on larval development or feeding.[30] Since we did not investigate the chirality of linalool identified in our experiments it is not possible to predict the likely effect of increased emission upon insect behavior.

In contrast to undamaged plants, stress treatment by leaf wounding and JA application in *P. putida*-colonized plants resulted in augmented emissions of nearly all volatiles tested, except for the green leaf volatile (*Z*)-3-hexenal (**Fig. 1**). These priming effects were statistically significant in at least one of the two experiments performed. Hence, root colonization by *P. putida* KT2440 appears to prime emission of stress-inducible aromatic and terpenoid volatiles from shoots. Why stress-induced emission of (*Z*)-3-hexenyl acetate was not primed by *P. putida* remains unclear. However, Maize plants primed by caterpillar herbivory show a similar pattern of aromatic and terpenoid volatile potentiation with no effect upon green leaf

volatiles. This suggests a similar mechanism between *P. putida*-induced defense priming and priming following exposure to herbivore-induced volatiles from neighboring plants.

To characterize defense priming in Maize in relation to benzoxazinoid-dependent root colonization by *P. putida*, we quantified stress-inducible gene transcription in control- and *P. putida*-treated plants of the benzoxazinoid-producing *BX1 igl* line and the BX-deficient *bx1 igl* line. Similar to stress-induced emission of aromatic and terpenoid volatiles (**Fig. 1**), basal levels of transcription of JA-dependent *MPI* and *SerPIN* were not directly influenced by the presence of *P. putida* (**Fig. 2**). Leaf wounding in combination with JA application resulted in transcriptional induction of both *MPI* and *SerPIN,* and was of similar intensity in both genotypes tested, suggesting that benzoxazinoids do not play a direct role in transcriptional activation of JA-dependent genes. Furthermore, stress-inducible *MPI* expression in both Maize genotypes was not influenced by *P. putida* root colonization. Hence, systemic defense priming by *P. putida* has no influence on the transcriptional responsiveness of *MPI* gene. This also resembles the response to herbivore-induced volatiles in Maize, where the *MPI* gene remained unresponsive to priming treatment.[21] In contrast, stress-inducible transcription of the *SerPIN* gene was strongly augmented in *P. putida* treated *BX1 igl* plants, while there was no evidence for such transcriptional gene priming in *bx1 igl* plants. Hence, the host plant's ability to synthesize benzoxazinoids determines *P. putida*-induced defense priming in the leaves.

Considering that root exudation of benzoxazinoids recruits *P. putida* to the rhizosphere,[16] our results suggest that benzoxazinoid-dependent root colonization by *P. putida* is important for aboveground defense priming in the host plant. It is, however, also possible that root-exuded benzoxazinoids exert an additional influence on *P. putida* physiology than simply stimulating chemotaxis and root colonization. For instance, benzoxazinoids may induce bacterial production of ISR-eliciting determinants in the rhizobacteria. A third explanation for benzoxazinoid-dependent defense priming by *P. putida* could arise from differences in defense
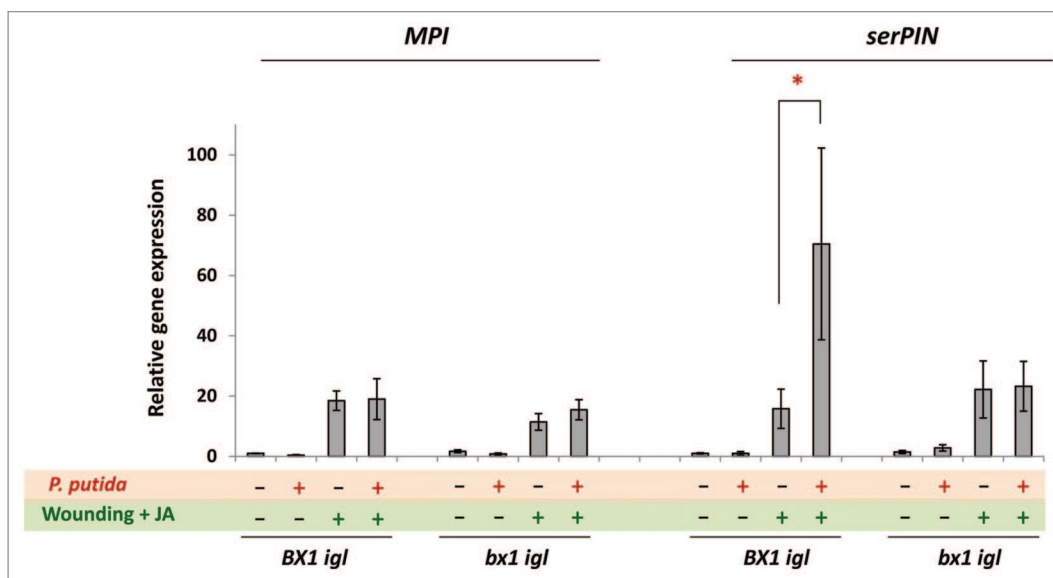
Figure 2. Transcription of JA-responsive genes in maize leaves of benzoxazinoid-producing (*BX1 igl*) and benzoxazinoid-deficient (*bx1 igl*) maize after root-colonization by *P. putida* KT2440 and subsequent leaf wounding and JA application. Shown are means of relative gene expression (± SEM; n = 6) at 8 h after wounding and JA application. Values are normalized to the average expression value of intact, control-treated *BX1 igl* plants. The asterisk indicates a statistically significant difference between the bacterized and non-bacterized group (Student's t-test; p < 0.05).

physiology between benzoxazinoid-producing and benzoxaiznoid-deficient host plants. We have previously demonstrated that apoplastic accumulation of DIMBOA in Maize during initial stages of aphid feeding and fungal infection boosts callose deposition.[19] Although benzoxazinoid-producing and benzoxaiznoid-deficient Maize lines do not show differences in JA-dependent defense gene expression in the absence of *P. putida*, we cannot exclude that the development of a primed defense state upon root colonization by *P. putida* requires functional benzoxazinoid metabolism in the leaves. However, considering that belowground exudation of DIMBOA promotes root colonization by *P. putida*,[16] we propose that the lack of defense priming in benzoxazinoid-deficient Maize plants relies on activity of the bacterial partner.

In context of our previous findings on the role of benzoxazinoids in Maize-biotic interactions,[16,19] our study further justifies the conclusion that these secondary metabolites play an important regulatory role in below- and aboveground defense responses of Maize. The implication that belowground benzoxazinoids recruit bacteria that promote aboveground defense responsiveness has consequences at multiple trophic levels. Further support for this notion comes from reference 31, who demonstrated that root benzoxazinoids can be exploited by the specialist root herbivore *Diabrotica virgifera* to localize nutrient-rich crown roots, which, in turn, can alter defense responses aboveground.[23] Further research on the effects of root-exuded benzoxazinoids on communities of plant-associated microbes and arthropods is warranted to fully reveal the importance of benzoxazinoids in cereal-biotic interactions.

Materials and Methods

Biological material. The green fluorescent protein-expressing strain FBC004 was used for all experiments, which is a derivative of *Pseudomonas putida* KT2440. Bacteria were cultivated as described previously.[16] *P. putida*-induced priming of wound- and JA-inducible volatile emission was studied in the Maize cultivar Delprim, which is routinely employed to study herbivore-induced VOC emission due to a robust and relatively strong volatile response to wounding.[20] To determine the role of benzoxazinoids in *P. putida* KT2440-induced defense gene priming, benzoxazinoid-producing and benzoxazinoid-deficient mutant lines of Maize were used, derived from a cross between *bx1* single-mutant and indole-deficient *igl* mutant lines, as described by ref. 19. Because the *bx1* mutant produces residual amounts of benzoxazinoids due to a functional *Indole-3-Glycerol phosphate Lyase* gene (*IGL*),[19] comparisons were made between benzoxazinoid-producing *BX1* and benzoxazinoid-deficient *bx1* lines in the *igl* mutant genetic background (i.e., *BX1 igl* vs. *bx1 igl*).

Root inoculation with *P. putida* and plant cultivation. Maize seeds were pre-germinated in wetted Petri-dishes for 3–4 d in the dark. Bacterial root colonization was effected by gently shaking sprouting seeds for 30 min in a suspension of washed *P. putida* cells from an overnight culture as described by ref. 16. A second set of seedlings were shaken in the same manner in sterile salt solution (3.4 mM $NaHPO_4$; 2 mM KH_2PO_4; 0.9 mM NaCl; 0.9 mM NH_4Cl) to provide the non-colonized control treatment. Visual observation of the roots using epi-fluorescence microscopy confirmed that roots exposed to GFP-expressing *P. putida* FBC004 were extensively covered in a bacterial film. Sets of four seedlings were transferred to 80 mL pots containing compost and grown for 10 d under controlled conditions (25°C; 16:8 h light-dark cycles; 150 μE m^{-2} s^{-1}).

Collection and quantification of volatile organic compound emission. For each treatment (with or without bacteria), 12 plants in three pots were stimulated for JA-dependent VOC emission by

wounding the first three leaves at two separate sites, using 12-inch serrated dressing forceps dipped in a 100 μM jasmonic acid solution (Sigma-Aldrich; J2500). Similar numbers of plants of each treatment remained free of mechanical stress. VOC emission was measured by air-entrainment as described previously.[21,22] Potted plants were placed in air-tight glass vessels and charcoal-purified air was pumped through at a rate of 0.7 L min^{-1}. Air exiting the vessels was passed through a trap containing Porapak™ Q beads. After 24 h, volatile traps were removed and the absorbed VOCs were eluted with three sequential 750 μL washes of redistilled diethyl ether, spiked with 200 ng/mL tridecane as internal standard. VOCs contained in the eluent were then identified using gas chromatography coupled to mass spectrometry (GC-MS) using a capillary gas chromatography column (EC05, 30 min length, 0.25 mm i.d., 0.25 μm film thickness) directly coupled to a mass spectrometer (VG Autospec, Fisons Instruments). Ionization was performed by electron impact (70 eV, 250°C). The oven temperature was maintained at 30°C for 5 min, and then programmed to rise 5°C min^{-1} up to 250°C. Volatile quantities were estimated on the basis of the internal standard (tridecane). Tentative compound identities were based on comparison of mass spectra with existing databases, and were confirmed by comparison of retention indices and mass spectra of authentic standards.

Gene expression analysis. Plants for gene expression analysis were treated similarly to VOC analysis, but were not kept in glass vessels after treatment by wounding and JA. Four shoots per pot were collected from three pots per treatment at 8 h after induction treatment. RNA extraction and cDNA synthesis were performed as described previously.[23] Quantitative PCR (qPCR) analysis of transcript accumulation of the *Maize Proteinase Inhibitor* gene (*MPI*) and the *Serine Proteinase Inhibitor* gene (*SerPIN*) was performed using a Corbett Rotor-Gene-6000, using previously described DNA primers.[21] Two technical replicates of each sample were subjected to the qPCR reaction. PCR efficiency (E) of primer pairs were estimated from data obtained from multiple amplification plots using the equation $(1 + E) = 10^{\text{slope}}$. Transcript levels were calculated relative to the constitutively expressed *Actin-1* and *Glycerol phosphate dehydrogenase C* (*GAPC*) genes,[21] using the $2^{-\Delta\Delta Ct}$ method.[24,25] Gene expression levels were normalized to average expression levels in control-treated, unwounded *BX1 igl* plants.

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