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Research Article

Engineering Plant Synthetic Pathways for the Biosynthesis of Novel Antifungals

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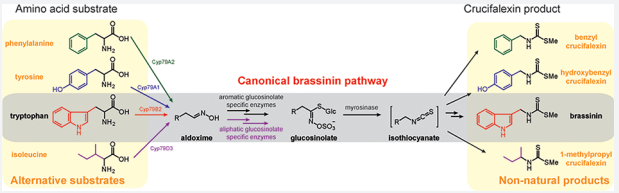
ABSTRACT: Plants produce a wealth of biologically active compounds, many of which are used to defend themselves from various pests and pathogens. We explore the possibility of expanding upon the natural chemical diversity of plants and create molecules that have enhanced properties, by engineering metabolic pathways new to nature. We rationally broaden the set of primary metabolites that can be utilized by the core biosynthetic pathway of the natural biopesticide, brassinin, producing *in planta* a novel class of compounds that we call crucifalexins. Two of our new-to-nature crucifalexins are more potent antifungals than brassinin and, in some instances, comparable to commercially used fungicides. Our findings highlight the potential to push the boundaries of plant metabolism for the biosynthesis of new biopesticides.



INTRODUCTION

Engineering of metabolic pathways in plants holds great promise for moving specialized metabolism from one plant to another.[1] Beyond direct pathway transfer there is the opportunity to engineer these pathways to generate new-to-nature compounds that are optimized for a particular application.[2] This pathway engineering approach is inspired by the success of natural product analogues and/or derivatives that have been generated for improved activity and/or efficacy in the clinic. Notably, many natural compounds are biosynthesized by plants as biopesticides, protecting them from various pathogens, albeit many times at much lower activity than traditional synthetic pesticides.[3] To explore the limitations of naturally occurring secondary metabolites, we sought to engineer novel biosynthetic pathways for new-to-nature biopesticides, inspired in structure by the natural metabolite brassinin.

The antipathogenic properties of cruciferous phytoalexins often have toxicity on plant cells, and so they are only produced in response to stress. However, phytoalexin pathways can be long, and *de novo* biosynthesis of a phytoalexin from primary metabolism may be too slow to stop pathogens.[4] To circumvent this problem, many plants constitutively accumulate phytoanticipins, nontoxic phytoalexin precursors which are quickly converted to phytoalexins as part of the stress response. In crucifers, the most common phytoanticipins are glucosinolates, biologically inert metabolites with a variable side chain on an imidothioic acid derivative, with the sulfur bound to glucose and the nitrogen bound to sulfate.[5] When the plant is under pathogen stress, or is physically damaged, the sugar of the glucosinolate is cleaved by a specific β-glucosidase enzyme, called myrosinase. At neutral pH, the aglucone forms a thiocyanate (pH > 7) or, more commonly, an isothiocyanate.[6] In *Brassica* species and a handful of others, indole isothiocyanates can be further modified into the dithiocarbamate containing molecule brassinin.[4,7]

Brassinin is an indole-sulfur phytoalexin found in several cruciferous crop species (e.g., *Brassica rapa*, *Brassica oleracea*) involved in plant disease resistance against various plant pathogens.[8] As with many plant natural products, brassinin has long been studied for its anticancer properties and has been found to inhibit the immune checkpoint enzyme indoleamine 2,3-dioxygenase.[9] Given its potential application in both agriculture and human health, the minimal set of enzymes necessary to engineer brassinin biosynthesis *in planta* has recently been elucidated.[4] In this pathway, tryptophan is first oxidized to its oxime via CYP79B2, and then tailored to indole glucosinolate. Subsequently, indole glucosinolate is activated to indole isothiocyanate and ultimately converted to the methyldithiocarbamate, brassinin (Figure 1a, Figure S1). Notably, chemically synthesized variations of brassinin have increased efficacy against cancer cell lines, suggesting that slight structural modifications may enhance the bioactive properties of the molecule.[9] In order to biologically produce brassinin alternatives, we aimed to expand the diversity of

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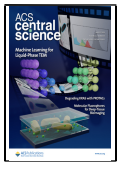

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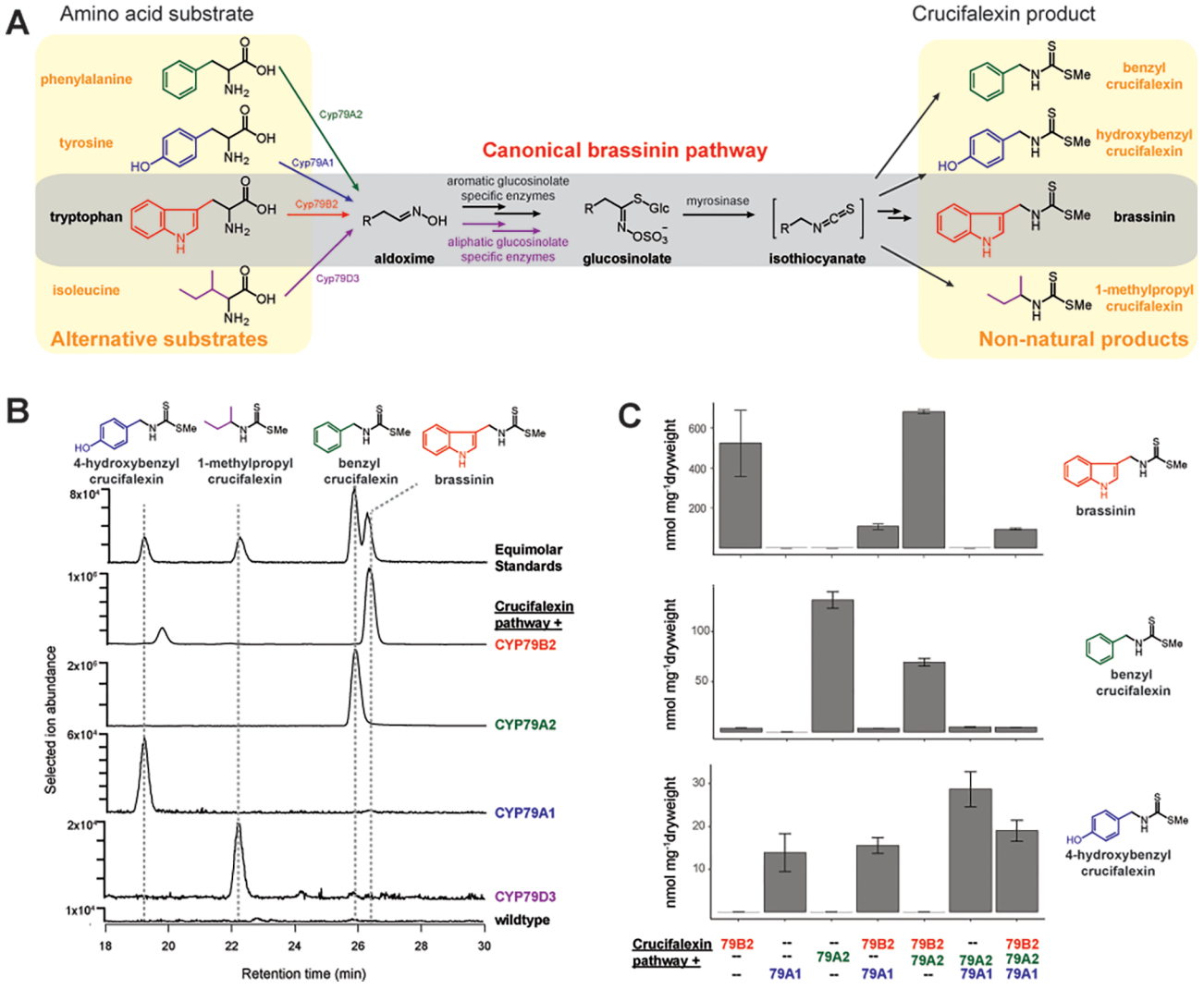

Figure 1. Engineering of crucifalexins from canonical amino acids. (a) Schematic of metabolic engineering for new-to-nature crucifalexins. For 1-methylpropyl crucifalexin, select enzymes in the crucifalexin pathway were taken from aliphatic ITC biosynthesis (purple arrows) rather than brassinin biosynthesis. There are several enzymes involved in the conversion of the isothiocyanate to brassinin; notably, there is a S-methyltransferase specific to this pathway. (b) LC/MS extracted ion chromatograph for crucifalexins accumulating in *N. benthamiana* plants engineered with the core crucifalexin pathway and one of several CYP79 enzymes or no heterologous enzymes (wild-type). Each chromatograph is normalized to its largest peak, with the exception of wild-type which is at the same scale as CYP79D3. The following ions were extracted for all chromatographs: *m*/*z* 130.0651, brassinin (dedithiocarbamate ion, molecule fragments in source, parent ion not detectable); 164.0562, 1-methylpropyl crucifalexin; 198.0406, benzyl crucifalexin; 214.0355, 4-hydroxybenzyl crucifalexin. (c) Accumulation of multiple crucifalexins in *N. benthamiana* expressing the core crucifalexin pathway along with CYP79A1, CYP79A2, or CYP79B2 individually, in pairs, or all together. Molecules were detected via LC/MS and quantified using the curve of synthetic standards.

substrates used by the brassinin biosynthetic machinery. To unlock the full biochemical diversity of the pathway, we tested various CYP79 homologs known to accept alternative amino acid substrates as the first committed enzyme in the pathway, effectively acting as the gatekeeper for pathway specificity.[10,11] Importantly, other CYP79 homologues have been characterized from disparate plant species for the oxidation of a range of amino acids.[12−14]

Since the discovery of glucosinolate genes, researchers have engineered their biosynthesis in the native plant and transferred the biosynthesis to heterologous plant hosts with the eventual goal of improving crop nutrition and defense. The first efforts were in 1999, when Bak et al. engineered *Arabidopsis thaliana* to produce 4-hyroxybenzyl glucosinolate by expressing CYP79A1 from *Sorghum bicolor* in the plant, demonstrating the native promiscuity of the glucosinolate pathway.[10] CYP79A1 oxidizes tyrosine to 4-hydroxyphenylaldoxime, which is tailored through the *A. thaliana* glucosinolate pathway, even though this substrate is not naturally present in the plant. CYP79D2 was later introduced into *A. thaliana*, causing the plant to produce leucine- and isoleucine-derived glucosinolates.[11] While all of these glucosinolates are found in nature, they were previously unreported in *A. thaliana*. Researchers soon found that expression of these glucosinolates altered the immunity of *A. thaliana*. Plants overexpressing CYP79A1/2 were more resistant to common bacterial pathogen *Pseudomonas syringae*, but more susceptible to the crucifer specialist fungi *Alternaria brassicicola*. Similarly, isopropyl and methylpropyl glucosinolates produced by expression of CYP79D2 increased resistance to soft rot fungus *Erwinia carotovora*.[15] Although there has been significant research demonstrating the potential in engineering various

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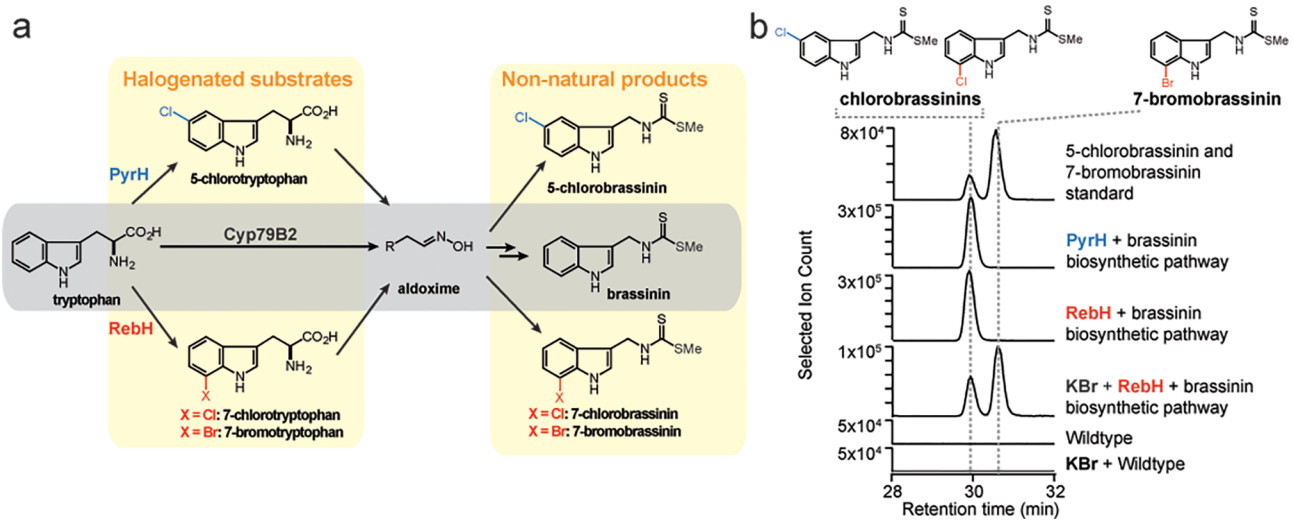

Figure 2. Engineering of halogenated brassinin derivatives. (a) Proposed halogenated brassinin pathway. Addition of halogenase reductase pairs PyrH/RebH or RebF/RebH produces 5-chlorotryptophan and 7-chlorotryptophan/7-bromotryptophan, respectively. (b) Accumulation of halogenated brassinin in engineered *N. benthamiana* plants. Selected ion chromatographs from LC/MS analysis of *N. benthamiana* leaf extracts expressing halogenases PyrH or RebH along with the reductase RebF and the brassinin pathway for production of chlorobrassinin or bromobrassinin (methylindole ions detected, m/z = 164.0262, m/z = 207.9756). Standard is mixture of 1 μM chemically synthesized 5-chlorobrassinin and 10 μM synthesized 7-bromobrassinin. Both RebH and PyrH produce a peak that matches an authentic synthetic standard of 5-chlorobrassinin mass and retention time. Given the specificity of RebH in the production of 7-chlorotryptophan previously reported in the literature [ref 19], we anticipate that the RebH product is likely the 7-chloro isomer that coelutes with the 5-chlorobrassinin standard.

glucosinolates, these approaches have not been well translated to downstream hydrolysis products of glucosinolates, which have novel bioactive properties.

RESULTS AND DISCUSSION

To test the capacity of the brassinin pathway for the biosynthesis of new-to-nature methyldithiocarbamates, we heterologously expressed the *B. rapa* pathway in *Nicotiana benthamiana* leaves, swapping tryptophan-specific CYP79B2 with phenylalanine-oxidizing CYP79A2 from *Setaria italica*, tyrosine-oxidizing CYP79A1 from *Sorghum bicolor*, or isoleucine- and valine-oxidizing CYP79D3 from *Lotus japonicus*.[12−14] Leaves expressing the engineered biosynthesis accumulated the intended new-to-nature molecules, indicating substantial substrate promiscuity across all the enzymes in the pathway (Figure 1b).

For simplicity, we will refer to this expanded class of cruciferous phytoalexins as "crucifalexins" and describe the crucifalexin pathway as the brassinin pathway without the R-group determining CYP79 enzyme. We have accordingly named these new-to-nature molecules benzyl crucifalexin, 4-hydroxybenzyl crucifalexin, and 1-methylpropyl crucifalexin. We also observed accumulation of a putative glycosylated version of 4-hydroxybenzyl crucifalexin with expression of CYP79A1 (Figure S2). Interestingly, we did not observe accumulation of valine-derived propyl crucifalexin with expression of CYP79D3, although we did observe the corresponding intermediate propyl isothiocyanate (Figure S3). In order to optimize metabolic flux through our various crucifalexin pathways, we tested the effect of various enzymes on overall product titers and found that TGG4, a promiscuous myrosinase from *A. thaliana*, had higher activity for new-to-nature products than the *B. rapa* myrosinase BABGLU.A (Figure S4).

As these novel molecules likely have different activities, it could be beneficial to engineer a suite of them into a single plant, mimicking the cocktail of phytoalexins produced simultaneously in crucifers like *B. rapa* and *Nasturtium officinale*.[7,8] Furthermore, the production of multiple compounds simultaneously may not only provide a defense against different pathogens but also potentially decrease the chances of evolving disease resistance to a single compound. Thus, we attempted to heterologously produce multiple new-to-nature crucifalexins in the same plant by transiently expressing the core pathway with pairwise combinations of *Br*CYP79B2, *Sb*CYP79A2, and *Si*CYP79A1, as well as all three together. When two or more CYP79s are expressed, all expected crucifalexins are detected, although their abundance drastically differs from when they are expressed individually (Figure 1c). This is especially apparent in combinations with CYP79A1. Surprisingly, although brassinin is the only "natural" molecule of the three, when all CYP79s were expressed together, 4-hydroxybenzyl crucifalexin production remained relatively fixed, while brassinin and benzyl crucifalexin levels dropped an order of magnitude. This finding suggests that enzyme promiscuity may be high enough to accept non-native substrate at significant rates. However, it is possible that production of all crucifalexins may be limited in this case, especially if the phenol group competitively inhibits one or more enzymes in the pathway.

With the inherent promiscuity of the downstream pathway, it is surprising that more crucifalexins are not naturally made. The lack of CYP79A1 and CYP79D3 homologues in sequenced genomes of brassinin-accumulating plants makes natural 4-hydroxycrucifalexin and 1-methylpropyl crucifalexin unlikely.[16,17] However, CYP79A2 homologues are found in several *Brassica* genomes, including *B. rapa*. Our previous work shows that the gene is expressed at low levels in *B. rapa* leaves accumulating brassinin, but no benzyl glucosinolate or benzyl crucifalexin could be detected in these tissues.[18] Furthermore, intermediate benzyl isothiocyanate does not accumulate in *B. rapa*, perhaps because it increases susceptibility to the crucifer

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pathogen *Alternaria brassicicola*.[15] The innovation of new metabolic pathways has largely been limited to evolving the existing set of genes found in a given plant genome. Thus, synthetic biology provides a means to bypass these natural constraints and engineer novel biosynthetic pathways that might have never evolved on their own.

Given the promiscuity of the crucifalexin pathway, we expanded our efforts to push the capacity of this pathway beyond canonical amino acids. Notably, synthetically prepared halogenated brassinin analogues have been shown to be more bioactive than brassinin and, thus, may have enhanced antifungal properties.[9] To produce halogenated brassinins *in planta*, we expressed the brassinin pathway in *N. benthamiana* along with a combination of bacterial halogenases/reductases: PyrH/RebF which produces 5-chlorotryptophan or RebH/RebF, which produces 7-chloro and 7-bromotryptophan[19] (Figure 2a). In these plants we observed that halogenated tryptophan was incorporated into the brassinin pathway. Leaves expressing the halogenated PyrH/RebF pathways accumulate 5-chlorobrassinin. Product formed from the RebH/RebF pathway is proposed to be 7-chlorobrassinin based on the precedence for RebH specificity. Notably, no bromobrassinin was observed in leaves. We reasoned that low levels of bromine in the plant would limit incorporation of this halogen onto tryptophan, and eventually brassinin. Since addition of exogenous bromine in hairy root cultures expressing RebH/RebF resulted in 7-bromotryptophan,[19] we watered RebH/RebF + brassinin expressing plants with potassium bromide (KBr), resulting in detectable levels of 7-bromobrassinin (Figure 2b). Although *in planta* production of chlorinated brassinins is clearly more practical, the accumulation of 7-bromobrassinin demonstrates the versatility of this pathway. The demonstration of engineering halogenated variants opens the door to future studies exploring the broader use of other newly discovered halogenases, such as the highly promiscuous RadH halogenase.[20]

Several cruciferous plants that produce brassinin often further tailor it to other bioactive phytoalexins, such as spirobrassinin and cyclobrassinin. There are two known enzymes that tailor brassinin: *Br*CYP71CR1, which makes spirobrassinin, and *Br*CYP71CR2, which produces cyclobrassinin.[18] Expression of CYP71CR1 or CYP71CR2 in plants engineered with the halogenated brassinin pathway yielded compounds with masses, MS-MS spectra, and isotope ratios matching 5- and 7-chlorospirobrassinin, 7-bromospirobrassinin (Figure S5), 5- and 7-chlorocyclobrassinin, and 7-bromocyclobrassinin (Figure S6). Production of engineered halogenated crucifalexins indicates that halogenated substrates do not severely inhibit any enzymes in the brassinin pathway. The expanded diversity of new substrates that can be utilized by the brassinin pathway underscores the potential to exploit enzyme promiscuity as a means to diversify and engineer novel molecules not found in nature.

Given the expansion of our new engineered crucifalexins, we sought to determine if any of our novel compounds could act as biopesticides against agriculturally relevant fungal pathogens. We chemically synthesized standards of benzyl crucifalexin, 4-hydroxybenzyl crucifalexin, 1-methylpropyl crucifalexin, and 5-chlorobrassinin (Figure S7) and assayed their inhibitory activity against the agriculturally relevant generalist pathogen *Botrytis cinerea*.[21] Benzyl crucifalexin, 4-hydroxybenzyl crucifalexin, and 1-methylpropyl crucifalexin have a lower inhibitory effect than brassinin (Figure S8). However, 5-chlorobrassinin outperformed brassinin and is comparable to pyrimethanil ($p < 0.05$)—an active ingredient in commercial pesticides—in rich media.[22] Furthermore, this IC_{50} is comparable to estimated levels of 5-chlorobrassinin produced heterologously, even without optimization of the pathway for this product (Figure 3). We estimate ~20 μM

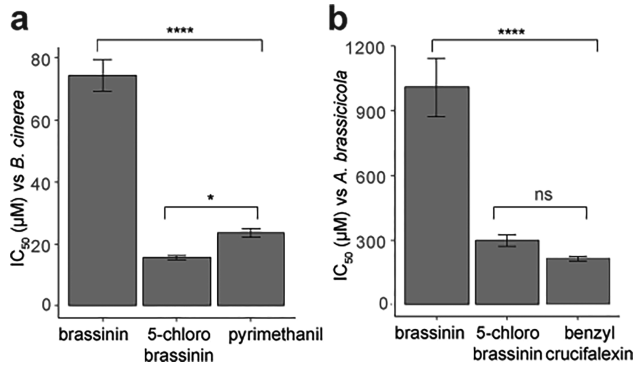

Figure 3. Inhibition of plant pathogenic fungi. IC_{50} of brassinin, new-to-nature crucifalexins, and commercial pesticide pyrimethanil against (a) generalist pathogen *Botrytis cinerea* and (b) crucifer specific pathogen *Alternaria brassicicola*. Inhibition determined by mycelial growth assay against DMSO control. Error bars represent standard deviation from a minimum of 11 biological replicates measured over two experimental trials. ns, not significant; * $p < 0.05$, **** $p < 0.0001$, one-way ANOVA.

production of 5-chlorobrassinin based on dry weight accumulation of 35 ± 13 μg/g as detected on LC/MS. Furthermore, we are producing similar levels of 7-chlorobrassinin (42 ± 4 μg/g dry weight, based on 5-chlorobrassinin standard) and 7-bromobrassin (37 ± 17 μg/g dry weight). It should be noted that the activity of pyrimethanil has been observed to be reduced in rich media given its function as an anilinopyrimidine inhibitor.[23] For additional context, another antifungal, fludioxonil, inhibits the growth of *Botrytis* isolates with IC50s on the order of 2.5 μM for many isolates.[24]

To demonstrate broad applicability of these novel metabolites, we also assayed their activity against the crucifer specialist *Alternaria brassicicola* (Figure S9). Both benzyl crucifalexin and 5-chlorobrassinin outperformed brassinin, with benzyl crucifalexin having the greatest inhibitory effect. Our findings demonstrate a means to produce new-to-nature crucifalexins that have improved efficacies over their natural analogue. Future efforts may optimize the large-scale production of these new compounds or directly engineer our synthetic metabolic pathways into crop plants as a complete or partial substitute to externally applied pesticides. Here, we present proof of concept that these new synthetic pathways can be ported and modified to produce new-to-nature compounds; however, there are several challenges that will be faced in the ultimate goal of being deployed in stable transgenic lines. Specifically, there are a minimum of eight genes that would need to be introduced into heterologous plant systems, requiring optimization of expression levels and accumulation. Moreover, there are several hurdles associated with the production of such glucosinolate-derived compounds which may necessitate proper compartmentalization. Our findings provide new targets for such long-term stable engineering efforts in the future.

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Modern agricultural practices are heavily dependent on the use of external pesticides, as three billion kilograms of pesticide are utilized annually.[25] Pesticide runoff can result in environmental and human health impacts,[26] which might be addressed by the direct production of biopesticides in crops. By combining pathways from different organisms, we rationally designed and reconstituted synthetic metabolic pathways for the production of a suite of new-to-nature small molecules. The antifungal activity of the engineered crucifalexins highlights the bioactivity of their shared dithiocarbamate group, which is similar to chemical moieties in many synthetic commercial pesticides like mancozeb and metam sodium;[8] however, their differences in activity levels demonstrate that the R-group is extremely important for their potency and has varied effects from pathogen to pathogen. By expanding the structural diversity of this class of molecules, we have engineered new derivatives with improved bioactivity over the natural metabolite, brassinin, with efficacies comparable to commercially used synthetic pesticides. Notably, specialized metabolic pathways differ in plants across phylogeny; our engineering approach enables the combination of diverse mechanisms for pathogen defense that have evolved independently in a way not accessible through breeding.

There are still notable challenges beyond our first proof-of-principle biosynthesis of novel compounds, such as how such engineered plants may interact with the environment and how to avoid resistance in pathogen populations, if engineered into stable transgenic plants. Related issues are encountered with the engineering of resistance genes for pathogen defense; here it is thought that stacking multiple genes can help prevent the development of resistance.[27] The development of tissue-specific and targeted approaches in engineered plants may also help address a number of these issues. Moreover, the use of the myrosinase introduces a natural trigger to produce the final molecule on demand in response to pathogen exposure or environmental stimuli. Nonetheless, the biological production of such compounds may provide a cost-effective means to scale the biosynthesis and extraction of new-to-nature products. Many of the halogenated derivatives we have biosynthesized may enhance the bioactive properties of brassinin, not only for disease resistance in plants but also against cancer cell lines, as has been shown in previous work.[9] Thus, our findings also provide a biological means to produce such compounds biologically, avoiding chemical synthesis. This work demonstrates the potential in harnessing synthetic biology to create new compounds with novel or improved bioactive properties in order to enhance the chemical arsenal of crop species for plant disease resistance.

METHODS

Materials and General Methods. Synthetic standards were prepared according to procedures documented in the literature: benzyl crucifalexin was synthesized from benzyl amine, 4-hydroxybenzyl crucifalexin from 4-hydroxybenzyl amine, and 1-methylpropyl crucifalexin from *sec*-butyl amine via a procedure from Pedras et al.[7]

5-Chlorobrassinin was synthesized from 5-chloroindole carboxaldehyde using a combined protocol from Griffieon et al. and Pedras et al.[7,28] 5-Chloroindole carboxaldehyde (690 mg, 3.76 mmol) was added to a mixture of hydroxylamine hydrochloride (366 mg, 5.27 mmol) and sodium acetate (463 mg, 5.65 mmol) in ethanol (10 mL) and stirred at 40 °C with reflux for 3.5 h. The product was concentrated under vacuum, extracted with ethyl acetate and water, and concentrated again under vacuum. The dried product was dissolved in glacial acetic acid (30 mL) with zinc dust (1.48 g) and stirred overnight at room temperature. The resulting suspension was filtered, washed and extracted with ethyl acetate, dried with sodium sulfate, and concentrated under vacuum to yield crude 5-chloroindole-3-methylamine (462 mg, 68% yield). The procedure was repeated to produce additional crude 5-chloroindole-3-methylamine (322 mg, 47% yield). 595 mg of crude product was added to trimethylamine (505 μL, 3.62 mmol) in pyridine (1 mL). Carbon disulfide (220 μL, 3.62 mmol) was added dropwise, and the solution was stirred at 0 °C for 20 min, at which point methyl iodide (226 μL, 3.62 mmol) was added dropwise, and the solution was stirred at 0 °C for another 30 min. The reaction was quenched with 1.5 M sulfuric acid (3 mL), extracted with diethyl ether, dried with sodium sulfate, and concentrated under vacuum. The product was purified via a flash silica column with ethyl acetate, and with another silica column (1:1 ethyl acetate:hexanes) to yield 5-chlorobrassinin (51.9 mg, 6% yield). 7-Bromobrassinin was synthesized via the same procedure using 7-bromoindole carboxaldehyde as the starting material.

All the synthesized compounds showed NMR and high-resolution mass spectrometry (HRMS) spectral data consistent with that reported in the literature. ^{1}H NMR spectra were acquired on a Varian 400 MHz spectrometer. HRMS data were obtained using an Agilent 6520 Q-TOF instrument, as described below. All chemicals were obtained from Sigma-Aldrich or Thermo Fisher Scientific, unless otherwise stated. Ultrapure water was generated by a Milli-Q system (EMD Millipore).

Cloning of the *B. rapa* Brassinin Pathway and CYP79 Genes. Phusion High-Fidelity DNA polymerase (Thermo Scientific) was used for all PCR amplification steps according to the manufacturer's instructions. All other enzymes used for cloning were purchased from New England Biolabs. Oligonucleotide primers were purchased from Integrated DNA Technologies. DNA excised from agarose gels was purified using the Zymoclean Gel DNA recovery kit (Zymo Research). *Escherichia coli* TOP10 cells (New England Biolabs) were used for plasmid isolation prior to transformation into other heterologous hosts. Plasmid DNA was isolated from *E. coli* cultures using the QIAprep Spin Miniprep kit (Qiagen). For *N. benthamiana* transient expression, all gene sequences were amplified from the *B. rapa* cDNA template with the exception of *At*TGG4 which was synthesized by Integrated DNA Technologies. Fusion CDS were generated using 2A peptides to express the core glucosinolate pathway. A *Br*SOT16−2A-*Br*UGT74B1−2A-*Br*SUR1 was expressed on the same plasmid under a tobacco mosaic virus 35S promotor. Similarly, a *Br*GGP1−2A-*Br*CYP83B1 fusion CDS was also expressed in the same way. BrAPK, AtTGG4, and BrDTCMT.a were expressed individually in pEAQ-HT. CYP79A2 was synthesized, inserted into pEAQ-HT[29] (KanR), and sequence verified by Gen9 based on its sequence in the *S. italica* genome. CYP79A1 (NCBI: XP_002466099.1), CYP79D2 (NCBI: AAF27290.1), CYP79D3 (NCBI: AY599895.1), RebH (NCBI: CAC93722.1), PyrH (NCBI: AY623051.1), and RebF (NCBI: BAC15756.1) were synthesized by Integrated DNA Technologies from sequences from *S. italica*, *S. bicolor*, *Morchella esculenta*, *L. japonicus*, *Streptomyces rugosporus*, and *Lechevalieria aerocolonigenes*.

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Transient Expression in *N. benthamiana*. pEAQ-HT and constructs were transformed into *Agrobacterium tumefaciens* (GV3101). Transformants were grown on LB plates containing 50 μg/mL kanamycin and 30 μg/mL gentamicin at 30 °C. Cells were removed with a sterile inoculating loop and resuspended in 1 mL of LB medium and centrifuged at 5000*g* for 5 min, and the supernatant was removed. The pellet was resuspended in 10 mM MES buffer, pH = 5.6, 10 mM $MgCl_2$, 150 μM acetosyringone, and incubated at room temperature for 1 h. *Agrobacterium* suspensions (OD600 = 0.1 for each strain) were infiltrated into the underside of *N. benthamiana* leaves with a needleless 1 mL syringe. Plants were grown 5−6 weeks under a 16 h light cycle prior to infiltration. Leaves were harvested 5 days postinfiltration, flash frozen, and stored at −80 °C for later processing. Three leaves per plant were infiltrated; a single biological replicate consisted of three pooled leaves from a single tobacco plant. Infiltrated leaf areas typically showed some signs of chlorosis or yellowing; leaves expressing GFP as a control also showed a similar phenotype.

Metabolite Extraction and LC/MS Analysis. For metabolite extraction, frozen samples were crushed with a pestle and lyophilized to dryness. The samples were subsequently homogenized on a ball mill (Retsch MM400) using 5 mm diameter steel beads, shaking at 25 Hz for 2 min. To the dry tissue, an 80:20 MeOH/H_2O (v/v) solution was added (20 μL/mg *N. benthamiana* tissue). Tissue extracts were heat treated at 65 °C for 10 min, spun at 15 000*g* for 10 min, passed through 0.45 μm PTFE filters, and analyzed (20 μL injection volume) by LC/MS.

LC/MS was performed on an Agilent 1260 HPLC instrument using a Gemini NX-C18 column (Phenomenex, 5 μm, 2 × 100 mm). Water and acetonitrile, each supplemented with 0.1% formic acid, were used as the mobile phase components, with a flow rate of 0.4 mL/min. For metabolite analysis of plant extracts, the following 43 min gradient was used (percentages indicate acetonitrile concentration): 3−50% over 30 min; 50−97% over 2 min; 97% for 3 min; 97−3% over 1 min; 3% for 5 min. Column eluent was monitored by an Agilent 1260 diode array detector followed by an Agilent 6520 Accurate-Mass Q-TOF mass spectrometer with an ESI source (parameters: mass range, 50−1200 m/z; drying gas, 350 °C, 11 L/min; nebulizer, 35 psig; capillary, 3000 V; fragmentor, 150 V; skimmer, 65 V; octupole 1 RF Vpp, 750 V; 1000 ms per spectrum). MS data were collected in either positive or negative ion mode (in the case of glucosinolates) and analyzed using MassHunter Qualitative Analysis software (Agilent). Phytoalexin content was calculated from the integrated peak area of a selected ion chromatogram (extracted using a ± 100 ppm window around the theoretical exact mass) and comparison to a standard curve. The first minute of each run was discarded to avoid salt contamination of the MS apparatus. For tandem mass spectrometry (MS/MS) analysis, 5, 10, 20, and 40 V collision energies were used with an m/z window of 4 centered on the m/z analyzed. Standard curves for brassinin, benzyl crucifalexin, 4-hydroxybenzyl crucifalexin, 1-methylpropyl crucifalexin, 5-chlorobrassinin, and 7-chlorobrassinin based on ion abundance were constructed and used to quantify amounts produced in *N. benthamiana*.

Fungal Mycelia Growth Inhibition Assay. Fungal growth inhibition assays were performed following a similar protocol to Pedras et al.[7] Spores of *Botrytis cinerea* SF1 or *Alternaria brassicicola* FSU218 were grown on PDA at room temperature in the dark for 3 days (*B. cinerea*) or 7 days (*A. brassicicola*). Serial dilutions of phytoalexins were made in DMSO and added to warm potato dextrose agar to final concentrations of 20, 50, 100, 200, and 500 μM and poured into 6-well plates (2 mL/well). The same procedure was used for pyrimethanil containing media, but with concentrations of 0.1, 1, 10, 25, 50, and 100 μM. 5 cm circular plugs of growing fungal mycelia were transferred to cooled plates and grown at room temperature in the dark for 24 h (*B. cinerea*) or 75 h (*A. brassicicola*). At this time the longest diameter of fungal growth was measured and compared to plugs grown on PDA with 1% DMSO to calculate percent growth inhibition. IC_{50} values and significance were calculated from percent growth inhibition of all replicates using a nonlinear regression model in GraphPad Prism.

Safety Statement. No unexpected or unusually high safety hazards were encountered with the reported work.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acscentsci.0c00241.

Additional data and figures including LC/MS data, NMR spectra, and inhibitory assay data (PDF)

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Notes

The authors declare the following competing financial interest(s): J.D.K. has a financial interest in Amyris, Lygos,

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