Biosynthesis of cabbage phytoalexins from indole glucosinolate

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Edited by Jerrold Meinwald, Cornell University, Ithaca, NY, and approved January 4, 2017 (received for review September 19, 2016)

Brassica **crop species are prolific producers of indole–sulfur phytoalexins that are thought to have an important role in plant disease resistance. These molecules are conspicuously absent in the model plant *Arabidopsis thaliana*, and little is known about the enzymatic steps that assemble the key precursor brassinin. Here, we report the minimum set of biosynthetic genes required to generate cruciferous phytoalexins starting from the well-studied glucosinolate pathway. In vitro biochemical characterization revealed an additional role for the previously described carbon–sulfur lyase SUR1 in processing cysteine–isothiocyanate conjugates, as well as the *S*-methyltransferase DTCMT that methylates the resulting dithiocarbamate, together completing a pathway to brassinin. Additionally, the β-glucosidase BABG that is present in *Brassica rapa* but absent in *Arabidopsis* was shown to act as a myrosinase and may be a determinant of plants that synthesize phytoalexins from indole glucosinolate. Transient expression of the entire pathway in *Nicotiana benthamiana* yields brassinin, demonstrating that the biosynthesis of indole–sulfur phytoalexins can be engineered into noncruciferous plants. The identification of these biosynthetic enzymes and the heterologous reconstitution of the indole–sulfur phytoalexin pathway sheds light on an important pathway in an edible plant and opens the door to using metabolic engineering to systematically quantify the impact of cruciferous phytoalexins on plant disease resistance and human health.**

plant natural products | *Brassica rapa* | metabolic engineering | dithiocarbamate

Cruciferous vegetables (family Brassicaceae) are widely grown agricultural crops that have notable traits in plant disease management (1, 2), soil ecology (3, 4), and human health (5, 6). For example, the incorporation of *Brassica* seed meal into soils can durably suppress apple replant disease (3). In addition, high consumption of cruciferous vegetables is associated with lower risk of human diseases, such as lung and colorectal cancers (5). Small molecules produced by crucifers, especially the glucosinolate family of natural products, have been implicated in these beneficial effects on both agriculture and human health. For example, accumulated evidence for the link between certain glucosinolates and cancer prevention has motivated an intensive breeding campaign to produce *Brassica* varieties that contain elevated levels of these molecules (7). These plants are currently commercially available and the subject of major human dietary intervention studies (8), one of the first of its kind where the effect of altered metabolite content in an edible plant on human disease prevention is being investigated.

Brassica species also produce high levels of a second family of molecules all proposed to originate from indole glucosinolate (Fig. 1). These compounds, called indole–sulfur phytoalexins, are a hallmark of the Brassicaceae with different subsets produced by different edible crucifers (Fig. 1) (9). Like glucosinolates, indole–sulfur phytoalexins have been linked to plant disease resistance and are thought to modulate human health: pure brassinin displays low-micromolar activities against plant pathogens (EC_{50} = 10–140 μM for restriction of various *Alternaria* phytopathogen isolates) (10) and cancer cells (K_i = 28 μM for anticancer target IDO) (11). Despite the activities observed in vitro, it has been difficult to systematically test the specific role of these molecules in the context of agricultural crops without the ability to control the timing and quantity of compound production. However, knowledge of a complete set of biosynthetic genes would enable engineering of these pathways into noncruciferous plants (e.g., soy or corn) and/or targeted mutagenesis in *Brassica* species for quantitative mechanistic studies.

Brassinin occupies a pivotal node in the proposed biosynthesis of indole–sulfur phytoalexins, where detailed labeling studies (Fig. 1) suggest that over 30 compounds arise from oxidative tailoring and rearrangement of this parent scaffold (9, 12). Labeling studies (13, 14) also indicate that brassinin itself is derived from indole glucosinolate via the activated product indole isothiocyanate, formed by the action of myrosinases (a class of thiospecific glucohydrolases) (Fig. 1). However, the complete set of enzymes that link indole glucosinolate to brassinin—which would be critical for metabolic engineering of indole phytoalexin biosynthesis—has not been described. Notably, although the biosynthetic pathway of glucosinolates and their activation during the innate immune response have been characterized in the model plant *Arabidopsis thaliana* (hereafter *Arabidopsis*) (15, 16), unlike other crucifers *Arabidopsis* does not produce brassinin nor its derived phytoalexins (17, 18).

As an important step toward elucidating the biosynthesis of indole–sulfur phytoalexins, we recently identified the first two dedicated enzymes, which differentially convert brassinin into cyclobrassinin and spirobrassinol (19). Here, we leveraged three orthogonal approaches to discover the key upstream biosynthetic steps that link indole glucosinolate to brassinin, namely: (*i*) chemical logic to predict biosynthetic enzyme families; (*ii*) pathogen-inducible pathway expression; and (*iii*) comparative genomics of *Arabidopsis* and *Brassica rapa*. We describe a divergent β-glucosidase and *S*-methyltransferase from *B. rapa* and report additional activities for previously identified glucosinolate biosynthetic genes that, together, allow for conversion of

Significance

Cruciferous vegetables—such as broccoli, mustard greens, and turnip—produce a variety of indole- and sulfur-containing chemicals when attacked by pathogens. These compounds, termed phytoalexins, are thought to serve as an important defense mechanism for the plant. Several phytoalexins have been shown to also have anticancer properties, raising the possibility that dietary exposure may be important to human health. Here, we report a minimal set of enzymes required to make brassinin, the parent cruciferous phytoalexin, from the well-studied glucosinolates. These genes enabled production of brassinin and related phytoalexins in tobacco leaves at levels comparable to those measured in cruciferous vegetables. The complete biosynthetic pathway to brassinin is critical for using metabolic engineering to characterize the biological role of indole–sulfur phytoalexins.

Author contributions: A.P.K. and E.S.S. designed research; A.P.K. performed research; A.P.K. and E.S.S. analyzed data; and A.P.K. and E.S.S. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

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This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1615625114/-/DCSupplemental.

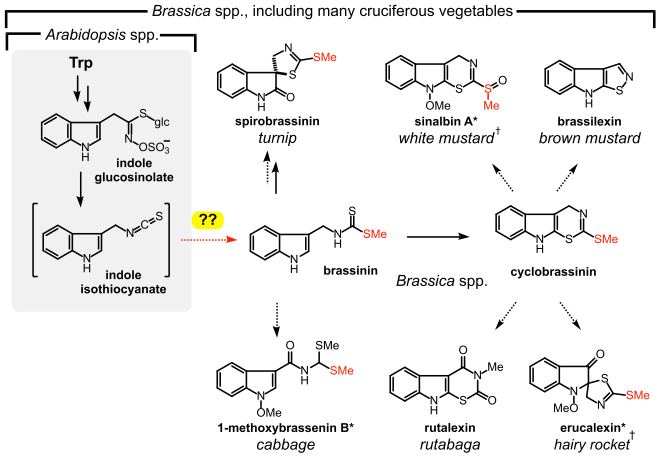

Fig. 1. Proposed biosynthetic pathway of indole–sulfur phytoalexins in cruciferous plants. Enzymes conserved between *Arabidopsis* and *Brassica* species synthesize indole glucosinolate from tryptophan (Trp). Following myrosinase-catalyzed activation of indole glucosinolate, an unknown reaction sequence generates brassinin from indole isothiocyanate (highlighted in yellow; formal methanethiol equivalent shown in red). Different *Brassica* crops accumulate a characteristic subset of the phytoalexins derived from brassinin. The names of representative phytoalexin producers are given in italics. Solid arrows represent known enzymatic reactions, and dashed arrows signify proposed biosynthetic relationships, many supported by previous isotope feeding studies (9). *The feeding studies suggest that 1-methoxybrassenin B, sinalbin A, and erucalexin are derived from the 1-methoxy derivatives of brassinin and cyclobrassinin. †White mustard and hairy rocket belong to the genera *Sinapis* and *Erucastrum*, respectively, which are each closely related to *Brassica*.

indole glucosinolate to brassinin through efficient formation and capture of an unprecedented dithiocarbamate intermediate. Critically, we define a set of enzymes sufficient to produce brassinin and other indole–sulfur phytoalexins in *Nicotiana benthamiana* directly from tryptophan, opening the door to using metabolic engineering to quantify the contributions of this class of metabolites to the valuable traits of cruciferous plants.

Results

Chemical Hypothesis and Bioinformatic Analysis to Identify Candidate Enzymes. We began our investigation by considering enzymatic reactions that could convert indole isothiocyanate into brassinin (Fig. 1). Indole isothiocyanate has not been directly observed because it readily decomposes (20), so efficient capture by a formal methanethiol equivalent appears to be required to direct this glucosinolate-derived metabolite toward phytoalexin biosynthesis. In a reaction sequence analogous to the glucosinolate biosynthetic pathway (Fig. 2*A*), it is plausible that the second sulfur atom of brassinin is introduced separately from the methyl group in two stages (Fig. 2*B*): (*i*) glutathione *S*-transferase (GST)-catalyzed addition of glutathione to indole isothiocyanate and (*ii*) enzymatic unmasking of the free thiol by the action of a γ-glutamyl peptidase (GGP) and a pyridoxal phosphate (PLP)-dependent carbon–sulfur lyase. The newly formed dithiocarbamate compound could then be captured by a dedicated *S*-adenosyl methionine (SAM)-dependent methyltransferase (MT). We also considered a reaction sequence that involves the incorporation of methanethiol as an intact unit (13), rather than the sequential addition of the thiol and methyl groups (*SI Appendix*, Fig. S1). Both of these proposed biosynthetic routes led us to concentrate our search on a C–S lyase and MT in *B. rapa* that could be involved in the conversion of indole isothiocyanate to brassinin.

In contrast to glucosinolates, indole–sulfur phytoalexins are produced only following pathogen elicitation, a feature of the innate immune response commonly regulated through the transcription of biosynthetic genes (21). We recently reported (19) a transcriptomic analysis of pathogen-treated *B. rapa* leaves using RNA sequencing (RNA-seq) and mined this dataset for candidate brassinin biosynthetic genes that are up-regulated following pathogen exposure relative to mock treatment. This analysis yielded several genes from each enzyme class predicted to participate in the conversion of indole glucosinolate to brassinin, including PLP-dependent enzymes and methyltransferases (Fig. 2*C*). To further refine the list of candidate genes, we used a comparative genomic approach because brassinin and downstream phytoalexins have not been detected in *Arabidopsis*. As a proof of concept, these three cumulative filters pinpoint the two CYP enzymes that we previously demonstrated

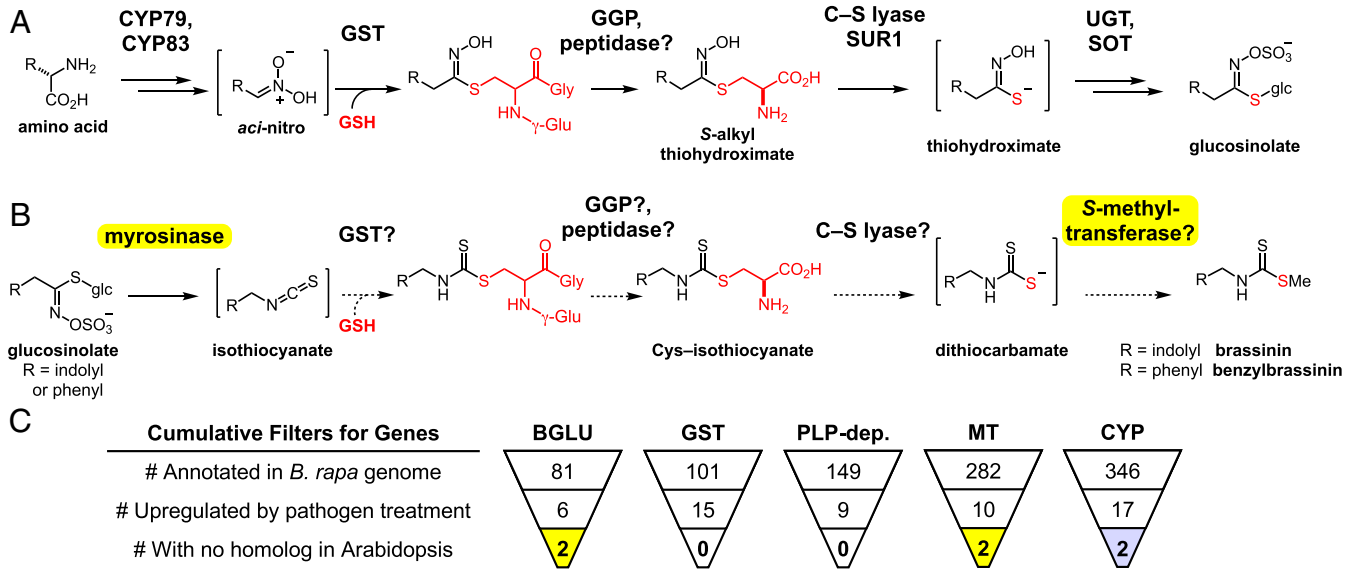

Fig. 2. Strategy to identify the missing enzymes in brassinin biosynthesis. (*A*) Well-characterized reactions in the biosynthesis of glucosinolates. (*B*) Chemical hypothesis of the enzyme families that could link indole glucosinolate to brassinin. (*C*) Bioinformatic analysis to identify candidate genes in brassinin biosynthesis, highlighted in yellow. Blue shading indicates previously validated genes for spirobrassinin and cyclobrassinin formation (19). The threshold for gene up-regulation was >1.8 $\log_2$ (fold change) for two pathogen elicitations compared with mock treatment (*SI Appendix, SI Materials and Methods*). BGLU, β-glucosidase; CYP, cytochrome P450; GGP, γ-glutamyl peptidase; GST, glutathione *S*-transferase; MT, methyltransferase; PLP-dep., pyridoxal phosphate-dependent enzyme.

tailor brassinin into cyclobrassinin and spirobrassinol (Fig. 2*C*) (19). Although the same filtering criteria yielded no candidates for the GST and PLP-dependent enzyme families, they did highlight MT and β-glucosidase (BGLU) candidates that are up-regulated in leaves following pathogen elicitation.

To test the candidate *S*-methyltransferases, we first needed to identify the enzymes that generate the predicted dithiocarbamate substrate (Fig. 2*B*), as this compound is likely an unstable pathway intermediate and cannot easily be prepared using chemical synthesis. According to our biosynthetic hypothesis, a Cys–isothiocyanate conjugate that is processed by a PLP-dependent C–S lyase could result in the requisite dithiocarbamate. Although we first looked for candidates that are unique to *Brassica*, each of the nine PLP-binding enzymes expressed concurrently with phytoalexin production has an ortholog in *Arabidopsis*. The most highly expressed among these genes is orthologous to *SUR1* (*SUPERROOT1*) in *Arabidopsis*, which encodes a C–S lyase involved in glucosinolate biosynthesis (22). Specifically, SUR1 has been shown to cleave *S*-alkyl thiohydroximates (Fig. 2*A*). We noted that the molecular structure of the predicted Cys–isothiocyanate intermediate bears a resemblance to *S*-alkyl thiohydroximates (Fig. 2*B*), suggesting that it could also serve as a substrate for SUR1.

The *SUR1* genes from *Arabidopsis* and *B. rapa* (*AtSUR1* and *BrSUR1*, respectively) were cloned, expressed individually in *Escherichia coli*, and purified by nickel-affinity chromatography. As expected, both AtSUR1 and BrSUR1 (89% amino acid identity) cleave the Trp-derived *S*-alkyl thiohydroximate in vitro, a key reaction previously described for indole glucosinolate biosynthesis (*SI Appendix*, Fig. S2). Next, we developed conditions to generate a benzyl analog of the predicted *SUR1* substrate in brassinin biosynthesis because, unlike indole isothiocyanate, benzyl isothiocyanate is stable, commercially available, and can be readily converted to the Cys–isothiocyanate conjugate in situ in the presence of cysteine. Upon the addition of AtSUR1 or BrSUR1 to this mixture, Cys–isothiocyanate is consumed and other peaks appear with masses that match dithiocarbamate derivatives (*SI Appendix*, Fig. S4). The catalytic properties of AtSUR1 and BrSUR1 were indistinguishable, and no conversion of Cys–isothiocyanate to dithiocarbamate was observed when SUR1 was first denatured by boiling (*SI Appendix*, Fig. S3). These results broaden the known substrate scope of SUR1 to encompass a Cys–isothiocyanate conjugate and suggest that SUR1 "plays double duty" in the biosynthesis of brassinin by acting on intermediates both upstream and downstream of indole glucosinolate.

A Dithiocarbamate *S*-Methyltransferase (DTC-MT) Catalyzes the Final Step in Brassinin Biosynthesis. Given the potential role of SUR1 in generating a dithiocarbamate derived from glucosinolate, we next sought to assay the candidate methyltransferases in vitro for production of the benzyl analog of brassinin. The two MT candidates (Fig. 2*C*) are paralogs in *B. rapa* that share 87% amino acid identity with each other. Phylogenetic analysis revealed that the enzymes belong to the SABATH MT family (23) and that they share ∼62% amino acid identity with farnesoic acid carboxyl-*O*-methyltransferase in *Arabidopsis* (*SI Appendix*, Fig. S9).

We expressed and purified the two putative methyltransferases DTC-MT.a (*Brara.B01660*) and DTC-MT.b (*Brara.G00303*) individually in *E. coli* for in vitro biochemical characterization. The addition of DTC-MT.a and SAM to a reaction mixture containing benzyl glucosinolate, commercial myrosinase, cysteine, and SUR1 results in disappearance of the dithiocarbamate and accumulation of benzylbrassinin (Fig. 3). In contrast, with DTC-MT.b, the dithiocarbamate persists and benzylbrassinin accumulates to ∼5% the level observed with DTC-MT.a (*SI Appendix*, Fig. S4). Efficient production of an *S*-methyl dithiocarbamate by DTC-MT.a (named "dithiocarbamate *S*-methyltransferase") supports a key role for this enzyme in brassinin biosynthesis in *B. rapa*.

To test the in vitro activity of DTC-MT.a using the native brassinin pathway intermediates, we incubated indole glucosinolate together with the three enzymes, Cys, and SAM. Brassinin accumulates in these reactions, although a prominent side product is formed from the decomposition of indole isothiocyanate (*SI Appendix*, Fig. S5). These findings indicate that additional components, such as a GST, may be required to direct indole isothiocyanate toward phytoalexin biosynthesis in planta.

In Planta Screening Identifies a Brassinin-Associated β-Glucosidase (BABG). Our experiments show that a commercial myrosinase from *Sinapis alba*, SUR1, and DTC-MT are sufficient for in vitro conversion of indole glucosinolate to brassinin. However, for metabolic engineering of the pathway in a noncruciferous plant, cellular and subcellular colocalization of enzymes and metabolic intermediates must be considered. In *Arabidopsis*, classical myrosinases [synonymous with BGLU subfamily 7 that comprises the *TGG* (thioglucoside glucohydrolase) genes] (24) are expressed exclusively in specialized cell types and combine with glucosinolates

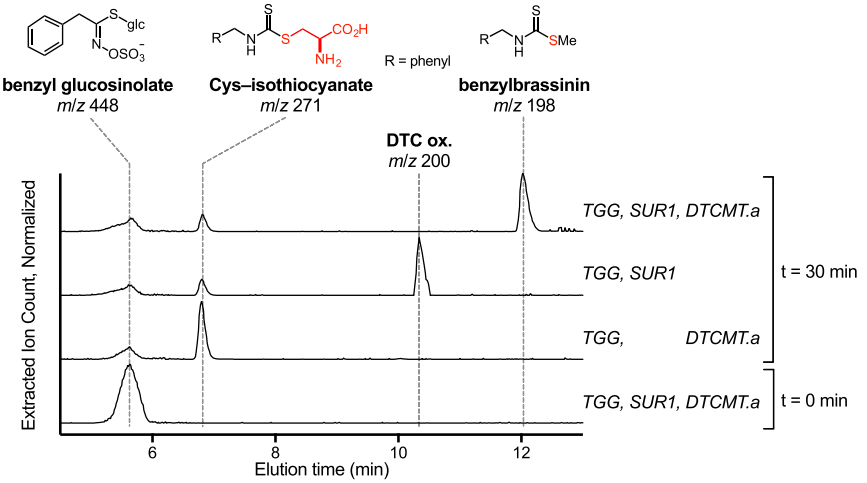

Fig. 3. In vitro reconstitution of benzylbrassinin formation from a glucosinolate. Enzymatic reaction products were analyzed using liquid chromatography coupled to high-resolution mass spectrometry (LC-MS). The catalytic activities of myrosinase TGG, C–S lyase SUR1, and dithiocarbamate *S*-methyltransferase DTCMT.a are depicted in Fig. 2*B*. The DTC ox. peak has the mass of a dithiocarbamate derivative (*SI Appendix*, Fig. S4).

only upon tissue damage to set off the "mustard oil bomb" (25). An atypical myrosinase PEN2 (*PENETRATION2*) metabolizes glucosinolates in intact *Arabidopsis* cells, although its subcellular anchoring to certain organelles may confine its catalytic activity (26, 27). We wondered whether a classical myrosinase, PEN2, or perhaps a novel type of myrosinase could suffice in engineering brassinin biosynthesis into a noncruciferous plant. Of the six *BGLU* genes up-regulated in our RNA-seq data set (Fig. 2*C*), none are members of the *TGG* subfamily, but this list does include an ortholog of *PEN2* and two genes with no ortholog in *Arabidopsis*. The two putative β-glucosidases, named "brassinin-associated β-glucosidase" [BABG.a (*Brara.D02695*) and BABG.b (*Brara.E00436*)], are paralogs that share 91% amino acid identity with each other. Phylogenetic analysis indicates that these enzymes belong to BGLU subfamily 4 (24) and that they share ∼56% identity with PEN2 (Fig. 4). Based on these bioinformatic considerations, we prioritized four BGLU genes for characterization: the two *BABG* candidates, *PEN2* from *B. rapa*, and the classical myrosinase *TGG4* from *Arabidopsis*.

To characterize the activity of these β-glucosidase genes in a plant host, we used transient expression in *N. benthamiana* (tobacco) leaves. Because tobacco does not natively synthesize glucosinolates, we simultaneously expressed eight genes from *B. rapa*, predicted to comprise the indole glucosinolate pathway (IG pathway) based on orthology to characterized enzymes in *Arabidopsis* (28, 29). This set of genes includes the SUR1 ortholog that we characterized in vitro. As expected, indole glucosinolate accumulates in tobacco leaves transiently expressing the IG pathway (Fig. 5 and *SI Appendix*, Fig. S7). When the classical myrosinase *TGG4* and *DTC-MT.a* were expressed in tobacco together with the IG pathway, the leaves contained ∼50% less indole glucosinolate compared with the IG pathway alone and had accumulated brassinin. The effect of expressing *BABG.a* or *BABG.b* is indistinguishable from that of *TGG4* (Fig. 5 and *SI Appendix*, Table S4), indicating that BABG.a/b too act as thioglucoside glucohydrolases. This result is surprising because until very recently (30) myrosinase activity had been thought to be rare outside of the *TGG* subfamily. In contrast, no glucosinolate conversion was observed for PEN2, possibly because this enzyme is not efficiently expressed in the transient assay or because its activity is biochemically or spatially safeguarded (30). Taken together, the two *BABG* genes encode myrosinases capable of activating indole glucosinolate for brassinin synthesis.

When the *DTC-MT.a* was excluded from tobacco transient expression of the IG pathway and *BABG.a*, brassinin was below the detection limit. Instead, a compound appeared with a mass that corresponds to indole dithiocarbamate *S*-glucoside (Fig. 5). Other studies have found that reactive metabolites are glucosylated in tobacco, presumably catalyzed by endogenous UDP-glycosyltransferases (UGT) (31). The dithiocarbamate methylation activity of *DTC-MT* genes observed in the tobacco transient expression system is in agreement with results obtained in vitro using purified enzyme.

Over 30 known cruciferous phytoalexins are proposed to be derived from brassinin or brassinin analogs (9, 32), and there is great interest in characterizing the biosynthetic pathway and biological function of each. To demonstrate the feasibility of using tobacco to test enzymes that modify brassinin en route to functionalized indole–sulfur phytoalexins, we added either *CYP71CR1* or *CYP71CR2* to the set of genes transiently expressed. Expression of either *CYP* resulted in the complete consumption of brassinin, coupled with the accumulation of spirobrassinin or cyclobrassinin, respectively (Fig. 5 and *SI Appendix*, Fig. S8). The amount of brassinin that accumulates in this tobacco system (∼400 pmol·mg^{-1} dry weight) is on par with the levels measured (19) in the native producer *B. rapa* (*SI Appendix*, Table S4).

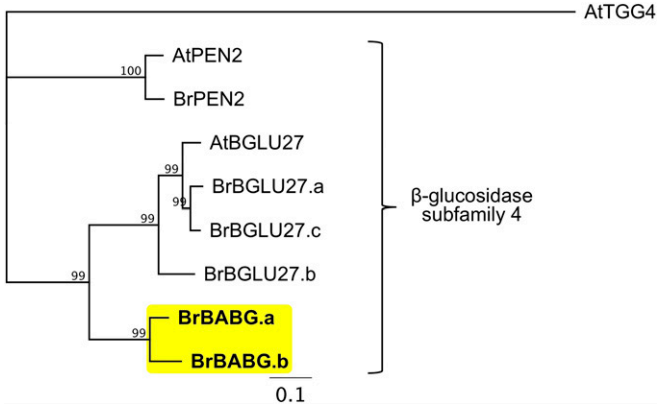

Fig. 4. Phylogenetic analysis of BGLU proteins. Members of the BGLU subfamily 4 from *Arabidopsis* (At) and *B. rapa* (Br) are shown, and the classic myrosinase AtTGG4 serves as an outgroup. Branch labels indicate consensus support based on Bayesian inference, and the scale bar represents the average number of amino acid substitutions per site. The brassinin-associated β-glucosidase (BABG) proteins are highlighted in yellow.

Discussion

Using chemical logic, transcriptomic analysis, and comparative genomics, we have identified enzymes that catalyze the conversion of indole glucosinolate to brassinin and reconstituted the biosynthesis of brassinin-derived phytoalexins in a heterologous plant. Biochemical analysis revealed a stepwise installation of the thiol and methyl groups of brassinin as a strategy to trap a reactive dithiocarbamate intermediate. Furthermore, two β-glucosidases that are distinct from the classical myrosinases are implicated in glucosinolate activation for brassinin synthesis. Taken together, this work provides tools to both engineer phytoalexin production and systematically probe the biological activities of these dietary metabolites.

The *S*-methyl dithiocarbamate moiety of brassinin is rare among natural products, but synthetic dithiocarbamates are widely used agricultural pesticides, amounting to over 10 million kilograms applied annually in the United States (33). The mechanisms of action of dithiocarbamate pesticides include the modulation of the cellular redox state and coordination with heavy metals, with the anionic compounds being more potent than the *S*-methylated forms (34, 35). In this investigation, we find evidence that an anionic dithiocarbamate is an endogenous metabolite of *Brassica* species and is the immediate biosynthetic precursor to brassinin. It is not known whether *DTC-MT* can accept synthetic dithiocarbamates as substrates, which may confer resistance toward these pesticides that are also toxic to plants (34).

Alternative routes to mobilize indole glucosinolate in response to pathogen attack have come to light. In *Arabidopsis*, the atypical myrosinase PEN2 defines one such pathway (16, 36), and the β-glucosidase PYK10 was recently reported to act in another (30). The strong induction of *BABG.a* and *BABG.b* expression following pathogen elicitation (*SI Appendix*, Table S1) and the functional characterization of *BABG.a* and *BABG.b* in tobacco (Fig. 5 and *SI Appendix*, Table S4) suggest that *BABG.a/b* could represent an additional pathway to activate indole glucosinolate for the synthesis of brassinin and its derived phytoalexins. However, additional experiments in *B. rapa* would need to be carried out to both more definitively link BABG.a/b to brassinin biosynthesis and rule out involvement of other myrosinases. Intriguingly, both the *Arabidopsis* and *B. rapa* genomes possess a phylogenetic intermediate between *PEN2* and *BABG*, named *BGLU27* (Fig. 4). Although *BGLU27* has very low expression in *Arabidopsis* (24), we detected the up-regulation of all three orthologous genes in *B. rapa* following pathogen treatment (Dataset S1). The consequences of *BGLU27* expression and its hypothetical role as a myrosinase remain to be studied.

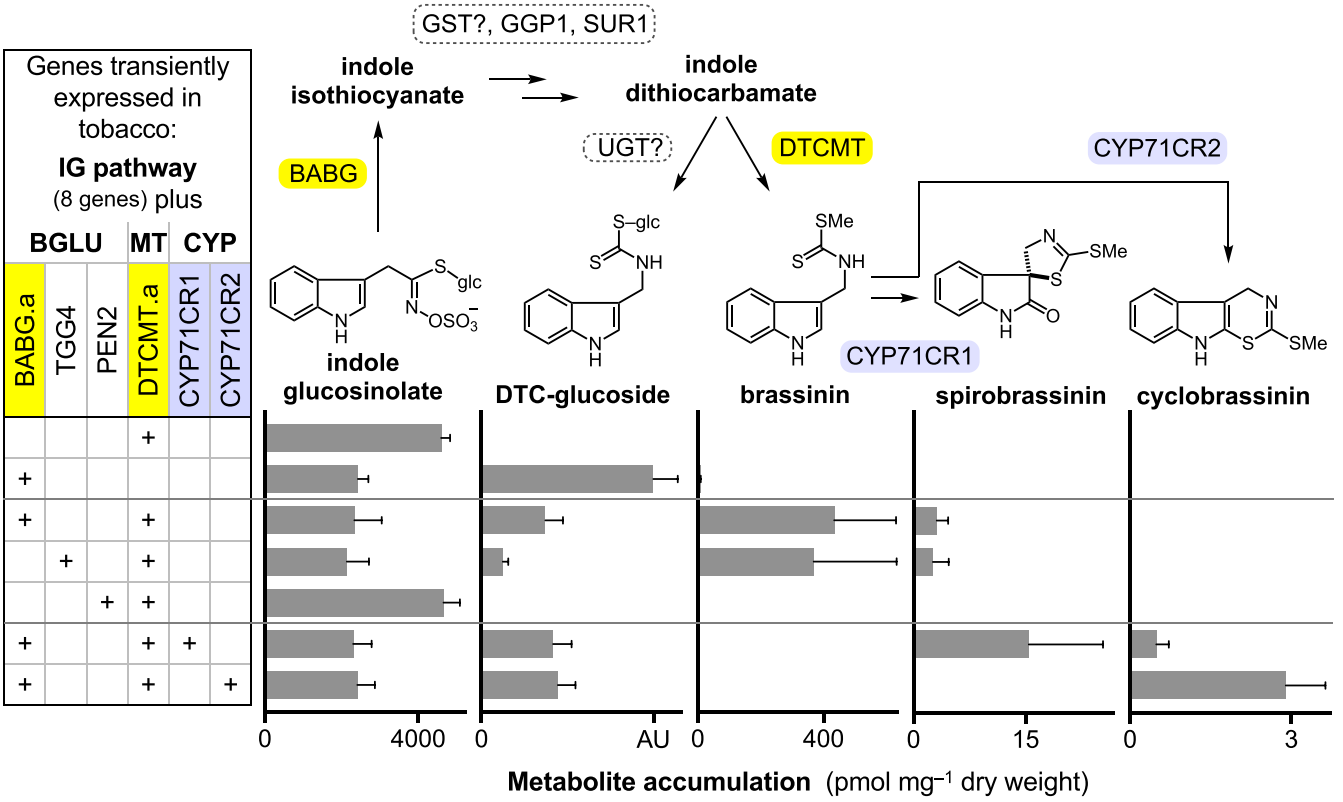

Fig. 5. Reconstitution of cruciferous phytoalexin biosynthesis in tobacco. Combinations of *B. rapa* genes were transiently expressed in the leaves of *N. benthamiana*. Metabolite accumulation in tobacco leaves was determined by LC-MS and plotted as mean ± SEM (n = 3). Indole isothiocyanate and indole dithiocarbamate are unstable intermediates that are not directly observed. The shadings of genes are the same as in Fig. 2. Enzymes enclosed in dashed lines are either those from the IG pathway acting twice or endogenous tobacco enzymes. IG pathway from *B. rapa*: CYP79B2, CYP83B1, GSTF9, GGP1, SUR1, UGT74B1, SOT16, and APK1.

The efficient pathway reconstitution in transiently transformed tobacco leaves suggests that a common enzyme cascade acts twice in brassinin biosynthesis. In particular, the capture of an electrophilic intermediate (indole isothiocyanate) by glutathione, followed by enzymatic processing to a reactive thiol (indole dithiocarbamate), closely parallels the conversion of *aci*-nitro compounds to thiohydroximates in glucosinolate biosynthesis (Fig. 2 *A* and *B*). The participation of peptidase GGP1 in processing the glutathione–isothiocyanate conjugate is inferred because a free primary amine on Cys is required for the catalytic mechanism of the C–S lyase (37). An additional peptidase to remove the Gly residue may be unnecessary if SUR1 cleaves the CysGly–isothiocyanate conjugate to generate the dithiocarbamate. The observation of some side products in the tobacco experiments suggests that additional components in *Brassica* species may channel metabolic flux, particularly in directing indole isothiocyanate–glutathione conjugation (*SI Appendix*, Figs. S7 and S8). We did not uncover any GSTs that met our criteria for brassinin-pathway specificity (Fig. 2*C*), but we cannot exclude the participation of one or more of the GSTs in *B. rapa* in brassinin biosynthesis.

Our results suggest the inability of *Arabidopsis* to synthesize or tailor brassinin is associated with the absence of three types of enzymes: BABG, DTC-MT, and the cytochrome P450 family 71 subfamily CR (CYP71CR). There is no evident genomic clustering of the genes involved in brassinin biosynthesis in *B. rapa*, and it remains mysterious what molecular evolutionary events led to the absence of orthologous genes in the *Arabidopsis* genome. *Arabidopsis* produces its own characteristic phytoalexins, including camalexin, whose biosynthesis has been engineered in vitro (38) and in tobacco (39). As more genome assemblies become available, evolutionary genomic approaches may be a powerful strategy to discover plant natural product biosynthetic pathways based on lineage-specific occurrence (40) and to discern the role of gene and genome duplications in diversifying plant specialized metabolism (41).

The elucidation of enzymes in brassinin biosynthesis opens the door to examining important questions about the biology of indole–sulfur phytoalexins. *Arabidopsis* engineered to produce cruciferous phytoalexins would enable the characterization of their physiological and ecological functions, for example, in interactions with beneficial and pathogenic microbes (42, 43); our preliminary work in tobacco suggests that metabolic engineering of the indole phytoalexin pathway could also be extended to other crop plants. Furthermore, an analysis of the subcellular localization of pathway enzymes may shed light on the importance of trafficking of defense chemicals and the possibility of metabolon formation (44) to channel reactive intermediates. Finally, reverse genetic techniques in *B. rapa* such as TILLING mutants (45) and CRISPR-mediated targeted knockouts could be used to establish whether the enzymes examined here constitute the major pathway in the native plant and what traits phytoalexins confer on *Brassica* crops.

Materials and Methods

Bioinformatic Analysis of Candidate Enzymes. The growth, phytoalexin elicitation, and RNA sequencing of *B. rapa* was previously reported (19) (GEO accession: GSE69785). Genes belonging to each enzyme family were identified in the *B. rapa* FPsc annotations (https://phytozome.jgi.doe.gov/) using PANTHER (v10) (46) classifications. The threshold for gene upregulation was >1.8 $\log_2$ (fold change) for two pathogen elicitations compared with mock treatment. The pathogen–up-regulated genes for each enzyme family are listed in Dataset S1, together with an associated bioinformatic summary.

Details of homology assignment to *Arabidopsis* are provided in *SI Appendix, SI Materials and Methods*.

Heterologous Production of SUR1 and DTC-MT Proteins in *E. coli*. The coding sequences of *BrSUR1.a*, *AtSUR1*, *DTCMT.a*, and *DTCMT.b* were cloned and individually expressed as C-terminal His_6-tagged proteins in *E. coli* BL21(DE3). The recombinant proteins were purified using nickel affinity chromatography.

In Vitro Assays of Recombinant Enzymes. For the chemoenzymatic synthesis of benzylbrassinin, the assay mixture was composed of *S. alba* myrosinase (30 mU/mL), purified BrSUR1 (100 μg/mL), purified DTCMT.a (40 μg/mL), SAM (1 mM), L-cysteine (1 mM), benzyl glucosinolate (200 μM), PLP (10 μM), and sodium phosphate buffer (50 mM, pH 7.5). Assays lacking BrSUR1 or DTCMT served as negative controls. Reactions were initiated by the addition of benzyl glucosinolate, incubated for 30 min at room temperature, and stopped by the addition of 1 vol of methanol. The composition of other in vitro enzyme assays is provided in *SI Appendix, SI Materials and Methods*.

LC-MS Analysis. Samples were separated by an Agilent 1260 HPLC using a Gemini NX-C18 column and analyzed by an Agilent 6520 Q-TOF mass spectrometer with an ESI source in positive ion mode.

Transient Expression of Biosynthetic Genes in *N. benthamiana*. The coding sequences of BrCYP79B2.a, BrCYP83B1, BrGSTF9.a, BrGGP1.a, BrSUR1.a, BrUGT74B1, BrSOT16.a, BrAPK1.a, AtTGG4, BrPEN2, BABGLU.a, BABGLU.b, DTCMT.a, DTCMT.b, CYP71CR1, and CYP71CR2 were cloned into the vector pEAQ-HT (47). These plasmids were transformed individually into *Agrobacterium tumefaciens* strain GV3101(pMP90) and used for transient T-DNA transformation. Eleven *Agrobacterium* strains were combined in equal densities for each inoculum (total OD_{600} of 0.6). When fewer than 11 genes were being characterized, *Agrobacterium* strains harboring either green fluorescent protein or β-glucuronidase cloned into pEAQ-HT were added to the inoculum to maintain consistent strain densities. Each combination of *Agrobacterium* strains was tested with three biological replicates at least three times on separate occasions, with similar results. Leaves were harvested 7 d postinfiltration, and the extracted metabolites were analyzed by LC-MS.

ACKNOWLEDGMENTS. We thank A. M. Calgaro, R. de la Peña, and R. J. Li for critical reading of the manuscript; C. R. Fischer for advice on mass spectrometry; the Stanford Functional Genomics Facility for RNA sequencing services; and J. C. Pires, M. B. Mudgett, and members of the E.S.S. laboratory for helpful discussions. This work was supported by National Institutes of Health Grant DP2 AT008321 (to E.S.S.). A.P.K. was supported by a National Defense Science and Engineering Graduate Fellowship.

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