Benzothiadiazole Conditions the Bean Proteome for Immunity to Bean Rust

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The common bean rust fungus reduces harvests of the dry, edible common bean. Natural resistance genes in the plant can provide protection until a fungal strain that breaks resistance emerges. In this study, we demonstrate that benzo(1,2,3)thiadiazole-7-carbothioic acid *S*-methyl ester (BTH) sprayed on susceptible beans induces resistance to common bean rust. Protection occurred as soon as 72 h after treatment and resulted in no signs of disease 10 days after inoculation with rust spores. By contrast, the susceptible control plants sustained heavy infections and died. To understand the effect BTH has on the bean proteome, we measured the changes of accumulation for 3,973 proteins using mass spectrometry. The set of 409 proteins with significantly increased accumulation in BTH-treated leaves included receptor-like kinases SOBIR1, CERK1, and LYK5, which perceive pathogens, and EDS1, a regulator of the salicylic acid defense pathway. Other proteins that likely contributed to resistance included pathogenesis-related proteins, a full complement of enzymes that catalyze phenylpropanoid biosynthesis, and protein receptors, transporters, and enzymes that modulate other defense responses controlled by jasmonic acid, ethylene, brassinosteroid, abscisic acid, and auxin. Increases in the accumulation of proteins required for vesicle-mediated protein secretion and RNA splicing occurred as well. By contrast, more than half of the 168 decreases belonged to chloroplast proteins and proteins involved in cell expansion. These results reveal a set of proteins needed for rust resistance and reaffirm the utility of BTH to control disease by amplifying the natural immune system of the bean plant.

Keywords: bean rust, BTH, mass spectrometry, *Phaseolus vulgaris*, proteomics, systemic acquired resistance, *Uromyces appendiculatus*

Bean rust fungi, namely the *Uromyces* spp., infect many varieties of commercially grown beans, causing farmers problems wherever rust appears. Bean rust disease is naturally

controlled by bean resistance (R) proteins that confer hypersensitive immunity to specific fungal isolates. For example, the Ur-3 and Ur-4 R proteins of the common dry edible bean *Phaseolus vulgaris* provide protection to numerous common bean rust strains (Hurtado-Gonzales et al. 2017; Lee et al. 2009). But not all are resisted. If the R protein combinations in the beans disagree with the rust isolates in the field, entire crop loss can occur during an outbreak.

R proteins function as receptors to specifically recognize, directly or indirectly, pathogen effector proteins that serve to disable the plant immune system to promote pathogen infection (Cook et al. 2015). Other types of receptors also exist within the plant immune system to recognize other types of pathogen proteins, carbohydrates, and metabolites (Cook et al. 2015). These receptor and recognition systems overlap and converge through salicylic acid (SA) and other hormonal signaling pathways that sustain, amplify, and transmit an immune response (Tsuda et al. 2009). In *Arabidopsis thaliana*, proteins EDS1 and PAD4 regulate SA responses from R proteins (Cui et al. 2017). Consequently, SA binding to NPR1/NPR3/NPR4 influences the expression of hundreds of genes needed for resistance (Ding et al. 2018). The expression output leads to protein and enzyme accumulation of the canonical pathogenesis-related (PR) proteins, the chitinases, glucanases, inhibitors, proteases, peroxidases, and defensins that target a broad spectrum of pathogens (van Loon et al. 2006). The SA signal output also leads to the synthesis of phytoalexins (toxins) (Nawrath and Métraux 1999) and the alteration of cell-wall composition including callose deposition and lignification (Tateda et al. 2014), all of which contribute to resistance to pathogens including bean rust and soybean rust fungi (Cooper et al. 2013; Lee et al. 2009).

Besides being involved with defense signaling at the site of infection, SA and its intracellular mobile form, methyl-SA, contribute to systemic acquired resistance (SAR), whereby infection at one leaf leads to increased SA accumulation and enhanced disease resistance at another leaf (Gaffney et al. 1993). SAR can be induced by topical application of SA, and this serves as a means of disease protection by preconditioning a plant to resist infection from a broader spectrum of pathogens than conferred by any single R protein (Klessig et al. 2018). Besides SA, there are other endogenous plant molecules that also transmit signals to induce SAR, including pipelicolic acid through a mechanism that is both SA-dependent and SA-independent (Bernsdorff et al. 2016; Chen et al. 2018; Hartmann et al. 2018). Other SAR inducers include dicarboxylic acid azelaic acid, abietane diterpenoid dehydroabietinal, and a glycerol-3-phosphate-dependent factor (Dempsey and Klessig 2012; Shah et al. 2014). Synthetic compounds like benzo(1,2,3)thiadiazole-7-carbothioic acid *S*-methyl ester (BTH) also can induce SAR (Görlach et al. 1996; Lawton et al. 1996). A

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commercial product sold to protect plants from myriad diseases, BTH may induce SAR through NPR1 or through multiple, overlapping pathways that include NPR1 (Delaney et al. 1994; Kohler et al. 2002).

Historical studies revealed that protein accumulation is an initial consequence of SAR (van Loon et al. 2006). Technology from the era of the early studies limited the identification to a few sets of PR proteins. Much about SAR has been learned since then, and we now know that there are several hundred to a thousand transcriptional changes associated with SAR (Blanco et al. 2009; Ding et al. 2018; Glazebrook et al. 2003; Krinke et al. 2007; Wang et al. 2005). Such a detailed proteomic picture, however, has not kept pace with the transcriptional landscape. So we aimed to use modern, high-throughput, quantitative mass spectrometry to measure protein accumulation changes associated with SAR. In this study, we examined the efficacy of BTH for inducing resistance to bean rust. We then evaluated the bean immune system after BTH application. Our examination of 3,973 proteins is the largest quantitative proteomic analysis, to date, for any plant undergoing SAR (Dong et al. 2016; Wu et al. 2015). The results describe a network of proteins that intersect SA defense pathways but also other hormonal pathways that appear to be involved in rust resistance.

RESULTS

Protection by BTH.

Previously, it was demonstrated that BTH application 7 days prior to rust challenge was better at protecting beans from common bean rust infection than the shorter waiting period of 3 days (Iriti and Faoro 2003). Knowing the need to protect plants as early as possible, we re-evaluated shorter waiting periods. In our preliminary tests, we observed no protection to rust when waiting 24 or 48 h after BTH application, but there was equivalent protection after waiting 72, 96, and 120 h when applying 0.7 mM BTH. We subsequently evaluated the 72-h waiting period in more detail. In three replicate trials, we found that BTH-treated leaves had no discernable pustules at 10 days postinoculation, whereas the water-treated controls were severely infected (Fig. 1A and B). In some cases, we observed only 1 or 2 pustules on BTH-treated leaves at 15 days postinoculation, but, by this time, the infected water-treated leaves died and abscised.

In contrast to BTH, 4.8-mM SA application and a 72-h waiting period reduced pustules only by 39% (Fig. 1C). Just as we observed for BTH, SA conferred no protection in preliminary trials with 24- and 48-h waiting periods, and the weaker SA protection observed at 72 h weakened further after waiting 96 h. We were not able to assess whether protection increased with greater concentrations of SA because the greater concentrations caused leaf burn (phytotoxicity). SA phytotoxicity from concentrations greater than 5 mM have been previously reported (Sillero et al. 2012). Depending on the plant, lower concentrations of SA also can be phytotoxic and not provide greater protection than what we observed (Kliebenstein et al. 2006; Sillero et al. 2012). Thus, BTH is preferable to SA because, even under heavy inoculum load, it induced near complete resistance in beans as soon as 3 days after application.

Quantitative mass spectrometry of BTH-treated bean leaves.

Wanting to know how BTH induces immunity in bean leaves, we used quantitative mass spectrometry to evaluate protein accumulation changes in control and BTH-treated leaves prior to rust inoculation. Proteins were isolated from leaves and were digested with trypsin. The peptides from each sample (three replicates of BTH-treated and three replicates of water-treated,

six samples total) were chemically labeled with a unique, isobaric tandem mass tag (TMT) (Isasa et al. 2015). The labeled peptide samples were combined and analyzed by mass spectrometry. Phosphopeptides were enriched from an aliquot of combined peptides and were analyzed as well. Overall, we identified 28,909 bean peptides, including 447 phosphopeptides. Parsimony, filtering, and grouping assigned those peptides to 3,973 quantified bean proteins (Supplementary Dataset S1). The normalized and scaled sum of the peptide TMT reporter ion signal-to-noise (S/N) ratios per protein were used for quantitation. The $\log_2$ fold changes of all proteins exhibited a normal distribution (Fig. 2A). Spearman ranked correlation clustering of the quantitative values revealed that the BTH-treated samples clustered together, pointing to the reproducibility of the experiments (Fig. 2B). Statistical analysis

Fig. 1. Rust disease on benzo(1,2,3)thiadiazole-7-carbothioic acid *S*-methyl ester (BTH)-treated and water-treated bean leaves. **A**, *Phaseolus vulgaris* variety Black Valentine was sprayed with 0.7 mM BTH and was inoculated with bean rust 72 h later (left) or was water-sprayed and inoculated (right). The photo was taken 10 days after inoculation. **B**, Average number of pustules per primary leaf ($n = 14$) for BTH-treated and water-treated plants at 10 days after inoculation. BTH-treated plants had zero visible pustules. **C**, Average number of pustules per primary leaf ($n = 20$) for 4.8 mM salicylic acid (SA)-treated and water-treated plants at 10 days after inoculation. There was a 72-h period between BTH or SA application and inoculation.

identified 577 proteins with significant accumulation changes arising from BTH treatment greater than or less than 0.35 ($\log_2$) with a false discovery rate (FDR) < 5% (Fig. 2C). Among these, there were 409 proteins with increased accumulation in BTH-treated leaves and 168 proteins with decreased accumulation. Of those, there were 31 phosphoproteins with increased accumulation (Table 1) and 14 phosphoproteins with decreased accumulation.

By categorizing proteins on their predicted subcellular localization, we found that the set of 409 proteins with increased accumulation had, in terms of percent distribution, twice the amount assigned to the cytoplasm and extracellular domain, and four to five times the amount localized to the endoplasmic reticulum (ER), nucleus, and peroxisome (Fig. 2D). This implied that the set of proteins that increased in accumulation was different than the set that decreased.

BTH increases receptor-kinase accumulation and affects multiple hormonal pathways.

In BTH-treated bean leaves, there were at least 120 proteins with increased accumulation with corresponding *A. thaliana* orthologs described in published literature as having direct and associated roles in immunity, disease resistance, and stress response. These proteins included numerous canonical PR proteins known to be induced by SA. Furthermore, in the set, there were two orthologs of EDS1, an ortholog of EDS1-interacting SAG101 (Feys et al. 2005), and two proteins orthologous to NDR1, which is required for SAR (Shapiro and Zhang 2001) (Table 2). Also found increased were SA-binding protein SABP2 (Kumar and Klessig 2003), DMR6 (Zhang et al. 2017b), and OM66, which regulate SA homeostasis and signaling (Zhang et al. 2014a). One of the proteins with one of the greatest accumulation changes was a carboxylesterase that belongs to the same hormone-sensitive lipase family as SABP2 (Kumar and Klessig 2003). These results indicate that BTH affected SA-regulated pathways.

Also among the increased proteins were those potentially associated with R protein immunity. One, an ortholog of SGT1B, had increased phosphorylation (Azevedo et al. 2002) (Table 1). Others appeared to be receptor-like kinases that likely function upstream of EDS1. These included an ortholog of SOBIR1, which activates a defense pathway suppressed by BIR1, a negative regulator of BAK1 (the brassinosteroid [BR] kinase that homodimerizes with the flagellin receptor FLS2) (Albert et al. 2015; Zhang et al. 2013). We also found orthologs of receptor-like kinase CERK1 critical for chitin elicitor signaling (Miya et al. 2007), its receptor-like kinase interactor LYK5 (Erwig et al. 2017), and two CIPP1 (CERK-interacting)

Fig. 2. Bean proteins quantified after treatment with benzo(1,2,3)thiadiazole-7-carbothioic acid *S*-methyl ester (BTH). **A**, Distribution of $\log_2$ average of normalized and scaled tandem mass tag (TMT) reporter ion S/N values for proteins from BTH-treated beans divided by the same for proteins from water-treated (control) beans. Bins are increments of 0.1. **B**, Spearman ranked correlation hierarchical clustering of the normalized and scaled TMT reporter ion summed S/N values for proteins from three biological replicates (126 = control R1, 127 = BTH R1, 128 = control R2, 129 = BTH R2, 130 = control R3, 131 = BTH R3). The scale bar shows that blue is the highest amount of relative accumulation (>35) and white is the lowest (<10). Clustering was performed with Treeview 3.0. **C**, Volcano distribution of fold-changes between bean proteins from BTH-treated and water-treated (control) leaves as a function of $-\log(\text{limma test } P \text{ value})$ for each set of reporter ion S/N values for each protein. Significantly different bean proteins ($n = 409$) with P values with $\leq 5\%$ false discovery rate (FDR) are in green and those with $> 5\%$ FDR are in black. **D**, Distribution of predicted cellular localizations for 409 proteins with increased accumulation in BTH-treated leaves and for 168 proteins with decreased accumulation in BTH-treated leaves.

protein phosphatases that balance immune signaling output from the receptors (Liu et al. 2018) (Tables 1 and 2). Altogether, we found 17 increased receptor-like kinases. It is possible that some of these are responsible for sustained phosphorylation for the 31 phosphoproteins that increased in accumulation, including a PEN3 ABC transporter ortholog (Table 1). In *A. thaliana*, PEN3 is crucial for transporting glucosinolates to resist fungi that penetrate cells (Clay et al. 2009). Along this line, there were increased amounts of at least 20 enzymes in the phenylpropanoid pathway (Fig. 3) that produces phytoalexins and cell wall–fortifying lignin to prevent rust infection when R-protein immunity is activated or when pathogen elicitors are applied (Bolwell et al. 1985; Cooper et al. 2013; Lee et al. 2009; Robbins et al. 1985).

Two of the increased receptor-like kinases are an ortholog of IOS1, which attenuates abscisic acid (ABA) responses (Hok et al. 2014) and interacts with CERK1 (Yeh et al. 2016), and the other is an ortholog of FERONIA, which is important for immunity but also suppresses jasmonic acid (JA) signaling (Guo et al. 2018) (Table 2). These increases of proteins that

function through ABA and JA hormonal pathways imply that BTH influences other hormonal responses in addition to SA. This appeared to be true with increased amounts of an ortholog of RAE1 that negatively regulates ABA by degrading the ABA receptor RCAR1 (Li et al. 2018) and BEN1 and TPR4, which positively increase levels of BR (Oh et al. 2014; Yuan et al. 2007) (Table 2). Meanwhile, the enzyme 1-aminocyclopropane carboxylate oxidase (ACO) that converts 1-aminocyclopropane-1-carboxylic acid (ACC) into ethylene (ET) was increased, as was EIN2, the protein that regulates ET signaling during pathogen defense (Tintor et al. 2013) (Table 2).

BTH alters cellular machinery.

The prediction of cellular localization revealed a greater distribution of extracellular proteins with increased accumulation (Fig. 2D). Many of these extracellular proteins include PR proteins. Because of the increase in secreted proteins, we assumed that there might be a concomitant increase in ER and vesicle-mediated transport machinery needed for protein

Table 1. Phosphoproteins with significantly increased accumulation in benzo(1,2,3)thiadiazole-7-carbothioic acid *S*-methyl ester (BTH)–treated beans

Protein	Annotation	Log ₂ fold change BTH/control	Predicted cellular localization	<i>Arabidopsis thaliana</i> ortholog	Function	<i>P</i> value	Phospho site
Phvul.001G040300.1.p	L-type lectin-domain receptor kinase	1.6	Cell membrane	AT5G10530	Immunity	4E-5	S366
Phvul.007G243200.1.p	ABC transporter G family member	1.3	Cell membrane	AT1G53390	Membrane transport	2E-4	S645
Phvul.008G212500.1.p	LYK5	1.2	Cell membrane	AT2G33580	Immunity	9E-5	S134
Phvul.010G024600.1.p	Ribosomal protein S8	1.1	Cytoplasm	AT5G59240	Ribosome	2E-5	S689; S690; S691; S873; S2946; S4457; S4457
Phvul.008G154713.1.p	Unknown	1.1	Golgi apparatus	No match	Unknown	3E-4	S530
Phvul.L001947.1.p	Inorganic phosphate transporter	0.99	Cell membrane	AT3G54700	Phosphate transport	1E-3	S100
Phvul.004G104600.1.p	SGT1B	0.92	Cytoplasm	AT4G11260	Immunity	5E-5	S98
Phvul.009G027000.1.p	Unknown	0.92	Nucleus	AT4G08550	Unknown	5E-5	S224
Phvul.001G172700.2.p	RS2Z32	0.88	Nucleus	AT3G53500	Splicing	2E-4	S18
Phvul.004G170800.1.p	NADP-dependent malic enzyme	0.80	Peroxisome	AT1G79750	Malate metabolism	1E-4	S198
Phvul.007G116000.1.p	F-box protein PP2-B10	0.78	Cytoplasm	AT2G02360	Immunity	1E-4	S13
Phvul.008G148700.1.p	Glucose-6-phosphate 1-dehydrogenase	0.74	Cytoplasm	AT5G40760	Immunity	2E-4	S285; S289
Phvul.007G066560.1.p	RS40	0.63	Nucleus	AT4G25500	Splicing	3E-4	S833; S850; S861
Phvul.010G145600.1.p	PEN3	0.61	Cell membrane	AT1G59870	Immunity	3E-3	S8
Phvul.006G087600.1.p	Glutamate decarboxylase	0.58	Cytoplasm	AT5G17330	Glutamate metabolism	2E-3	S10
Phvul.004G082700.1.p	PIP2;2	0.55	Cell membrane	AT2G37170	Water transport	5E-3	S405
Phvul.004G082894.1.p							
Phvul.008G155500.1.p	S/T protein kinase	0.53	Nucleus	AT5G40340	Flowering	8E-4	S115
Phvul.006G206300.1.p	CIPP1	0.52	Plastid	AT1G34750	Immunity	2E-3	S85
Phvul.003G294800.3.p	RBP47	0.51	Nucleus	AT3G19130	RNA processing	2E-3	S252
Phvul.011G026600.1.p	Translocon-associated protein	0.50	Cell membrane	AT2G16595	Protein processing in the endoplasmic reticulum (ER)	1E-3	S308
Phvul.001G186200.1.p	U2AF35B	0.46	Nucleus	AT5G42820	Splicing	2E-3	S120; S121
Phvul.001G098000.1.p	WD repeat	0.45	Nucleus	AT1G12910	Anthocyanin production	7E-3	S110; S200; S205
Phvul.007G270200.1.p	RSZ32	0.44	Nucleus	AT3G53500	Splicing	2E-3	S807
Phvul.001G077100.1.p	ABC family protein	0.44	ER	AT5G24810	Unknown	4E-3	S960; S963
Phvul.001G029800.1.p	PRP40A	0.44	Nucleus	AT1G44910	RNA processing	5E-3	S133; S139
Phvul.010G072400.1.p	Vesicle-associated protein 1-2	0.42	ER	AT2G45140	Vesicle mediated transport	6E-3	S173
Phvul.006G029300.1.p	CWC22	0.41	Nucleus	AT1G80930	Splicing	4E-3	S452
Phvul.005G181500.1.p	BRAP2 ring Zn finger	0.40	Cytoplasm	AT2G26000	Unknown	6E-3	T529
Phvul.003G098200.1.p	Pyruvate phosphate dikinase	0.40	Mitochondrion	AT4G15530	Pyruvate metabolism	6E-3	S4; S6; S8; T9
Phvul.003G219400.1.p	Lactate dehydrogenase	0.37	Cytoplasm	AT4G17260	Glycolysis	7E-3	S795
Phvul.007G149432.1.p	Cell division cycle 48	0.37	Cytoplasm	AT5G03340	Protein processing in the ER	7E-3	S317
Phvul.007G149366.1.p							

secretion. Indeed, there were 49 proteins with increased accumulation predicted to localize to the ER, whereas only four ER proteins decreased. These proteins include protein disulfide isomerases, glycosyltransferases, cytochrome P450 reductases, calreticulins, calnexins, heat-shock proteins, luminal-binding proteins, translocon SEC63, vesicle-associated proteins, and signal peptidases (Supplementary Fig. S1).

There also was a preponderance of nuclear proteins with increased accumulation (Fig. 2D). Among the nuclear proteins were at least 13 proteins attributed to pre-mRNA processing and transcript splicing, including orthologs of SCL30, SRZ22, CWC22, PRP40A, U2AF35B, RS40, RBP47, and RSZ32. The CWC22, PRP40A, U2AF35B, RS40, RBP47, and RSZ32 orthologs had increased amounts of phosphorylation as well (Table 1). Splicing regulation is controlled by phosphorylation and is known to influence immune response (Zhang et al. 2014b, 2017a).

Bean proteins with decreased accumulation include plastid proteins.

Of all 168 proteins with decreased accumulation arising from BTH application, half had predicted localization to the chloroplast (Fig. 2D). This included at least eight enzymes catalyzing the synthesis of chlorophyll and many other structural chloroplast proteins that compose the photosystems and thylakoid membranes (Supplementary Fig. S2).

Besides this effect on plastid proteins, there were reduced amounts of proteins that appear to function in cell-wall organization and expansion. Six of the top 10 proteins with the greatest reductions in accumulation are predicted to function at the cell wall or cuticle. There were at least 12 other proteins with functions that could be attributed to cell-wall organization. Furthermore, two proteins with some of the greatest reductions were orthologous to auxin-regulated FLA2 and patellin (Table 2). In *A. thaliana*, FLA2 associates with auxin exporter PIN1 (Titapiwatanakun et al. 2009) and *A. thaliana* patellin mutants have defects in cell development (Tejos et al. 2018). Furthermore, two homologs of ABCB1, another auxin exporter

(Noh et al. 2001), were reduced. These findings imply that auxin signaling was repressed, possibly to reduce cell expansion to promote lignification. Meanwhile, there were decreased amounts of an ortholog of CIPK3, which controls ABA responses to stress, including dehydration (Kim et al. 2003) (Table 2). Coinciding with this, there were decreased amounts of orthologs to ABA-regulated aquaporin PIP2;7 and PIP2;2 that are important for cell membrane water permeability during salinity stress (Boursiac et al. 2005; Jang et al. 2004; Pou et al. 2016).

Comparison with published gene expression and proteomics data.

Over the last 20 years, many laboratories have measured gene expression changes in *A. thaliana* treated with SA. By using BLAST to match *A. thaliana* genes to bean proteins, we compared some of the *A. thaliana* gene-expression data to the bean protein changes reported here. Using Affymetrix GeneChips representing 8,200 genes, Wang et al. (2005) measured 197 gene expression changes between *NPR1* transgenic and *npr1* mutant plants treated with SA for 24 h. Those authors hypothesized that the comparative analysis would refine the set of SAR responses controlled through NPR1. Of these gene expression changes, 20 correlated with the protein changes observed in BTH-treated beans. The overlap included EDS1 and LYK5 and proteins involved in protein processing at the ER. Using a different array of 24,576 *A. thaliana* gene-specific tags, Krinke et al. (2007) measured 792 gene expression changes in *A. thaliana* cell suspensions treated with SA for 4 h. There were 54 gene-expression changes that shared the same direction of change as the bean proteins. Among these proteins were DMR6, EDS1, PR4, PEN3, and FLA2. Blanco et al. (2009) used a cDNA array to measure changes in 18,981 genes between *A. thaliana* wild type and *npr1* mutants treated with SA for 2.5 h. They identified 217 expression changes, but only 18 overlapped with the bean protein changes, including CERK1, IOS1, and DMR6. More recently, Ding et al. (2018) used high-throughput cDNA sequencing to find 2,455 differentially expressed genes between *A. thaliana* wild type and

Table 2. Proteins with significantly altered accumulation in benzo(1,2,3)thiadiazole-7-carbothioic acid *S*-methyl ester (BTH)-treated beans

Protein	Annotation	Log ₂ fold change BTH/control	Predicted cellular localization	<i>Arabidopsis thaliana</i> ortholog	Function ^a	P value
Phvul.009G183000.1.p	EDS1	0.66	Mitochondrion	AT3G48080	Immunity/SA signaling	8E-4
Phvul.009G183100.1.p	EDS1	1.7	Peroxisome	AT3G48090	Immunity/SA signaling	7E-5
Phvul.004G020000.1.p	SAG101	1.5	Cytoplasm	AT5G14930	Immunity/SA signaling	6E-5
Phvul.001G194700.1.p	NDR1	1.7	Cell membrane	AT2G35980	Immunity/SA signaling	4E-4
Phvul.001G194600.1.p	NDR1	1.8	Cell membrane	AT2G35980	Immunity/SA signaling	7E-4
Phvul.003G248075.1.p	SABP2	0.79	Cytoplasm	AT2G23620	SA homeostasis	1E-3
Phvul.009G044600.2.p	DMR6	2.3	Cytoplasm	AT5G24530	SA homeostasis	2E-4
Phvul.003G256600.1.p	OM66	0.74	Cytoplasm	AT3G50930	SA homeostasis	2E-4
Phvul.003G295200.1.p	Carboxylesterase	3.3	Cytoplasm	AT3G48700	Hormone-sensitive lipase	4E-6
Phvul.009G171200.1.p	SOBIR1	1.3	Cell membrane	AT2G31880	Immunity	5E-5
Phvul.006G006700.1.p	CERK1	0.53	Cell membrane	AT3G21630	Immunity	1E-3
Phvul.002G209700.1.p	CIPP1	0.54	Plastid	AT1G34750	Immunity	2E-3
Phvul.005G164500.1.p	IOS1	2.1	Cell membrane	AT1G51800	Immunity/ABA signaling	8E-5
Phvul.008G030800.1.p	FERONIA	0.97	Cell membrane	AT3G51550	Immunity/JA signaling	4E-4
Phvul.003G032400.1.p	RAE1	0.43	Nucleus	AT1G80670	ABA signaling	3E-3
Phvul.008G076600.1.p	BEN1	2.0	Cytoplasm	AT2G45400	BR regulation	1E-4
Phvul.003G167500.1.p	TPR4	0.38	Cytoplasm	AT3G15880	BR regulation	6E-3
Phvul.003G020200.2.p	ACO	0.84	Cytoplasm	AT1G03400	ET biosynthesis	4E-4
Phvul.007G150600.1.p	EIN2	0.51	Cell membrane	AT5G03280	ET signaling	7E-3
Phvul.008G288800.1.p	FLA2	-1.0	Cell membrane	AT4G12730	Auxin transport	7E-5
Phvul.009G157900.1.p	Patellin	-0.96	Cytoplasm	AT1G72160	Auxin-mediated cell elongation	4E-4
Phvul.001G179300.1.p	ABCB1	-0.69	Cell membrane	AT2G36910	Auxin regulation	2E-4
Phvul.007G147400.1.p	ABCB1	-0.50	Cell membrane	AT2G36910	Auxin regulation	6E-3
Phvul.008G205700.1.p	CIPK3	-0.52	Cell membrane	AT2G26980	ABA regulation	9E-4
Phvul.009G118900.1.p	PIP2;7	-0.70	Cell membrane	AT4G35100	Water transport	5E-3
Phvul.004G082600.1.p	PIP2;2	-0.66	Cell membrane	AT2G37170	Water transport	2E-3

^a SA = salicylic acid, ABA = abscisic acid, JA = jasmonic acid, BR = brassinosteroid, and ET = ethylene.

npr1 mutants treated with SA for 1 h. Only 71 of those overlapped with the bean proteomics changes. The proteins included IOS1, DMR6, OM66, CIPP1, LYK5, NDR1, SOBIR1, SAG101, EDS1, CERK1, PEN3, CIPK3, and several proteins involved in ER protein processing and photosynthesis. From all four transcriptional studies, there were just two proteins, the CIPP1 protein phosphatase orthologs, that overlapped with our proteomics data. In *A. thaliana*, this phosphatase interacts with the chitin-elicitor receptor kinase CERK1 to regulate phosphorylation signaling output for disease resistance pathways (Liu et al. 2018). Altogether however, we found an overall lack of continuity between our proteomics data and published gene-expression data. We attribute this to imprecise matching between *P. vulgaris* and *A. thaliana* protein orthologs, possible differences between responses to BTH and SA application, differences between SAR in bean and *A. thaliana*, and a general lack of absolute congruence between gene-expression and protein-abundance data (Vogel and Marcotte 2012).

We also compared our data to 563 protein changes that we previously observed in beans genetically immune to rust infection (Lee et al. 2009). We found that the 51-protein overlap included not just PR proteins but also proteins involved in secretion and enzymes to produce phenylpropanoids. Furthermore, there was an overlap of proteins with reduced amounts of FLA2 and patellin, which together allude to an auxin response that may help promote lignification, and reduced amounts of PIP2;7, an ABA-regulated water transporter (Jang et al. 2004). There also was a corresponding reduction of chloroplast proteins. These results reveal that BTH alters the bean proteome similarly to a hypersensitive immune response to the bean rust fungus.

Evaluation of hormone and phenylpropanoid changes.

Our proteomics data reveal that BTH increased the accumulation of proteins in disease-resistance pathways that function through SA hormonal sectors. The proteomics data also implicate alterations in other hormonal pathways, such as those controlled or regulated by ET, ABA, and auxin. Furthermore, the increase in enzymes functioning in the phenylpropanoid pathway portends a possible increase in phenylpropanoids that lead to disease resistance. To test potential effects on hormone and phenylpropanoid production, we analyzed BTH-treated bean leaf extracts by mass spectrometry. The mass spectrometer was configured to specifically examine masses for SA, ACC, ABA, and auxin (indole-3-acetic acid). Phenylpropanoids *trans*-cinnamic acid, *p*-coumaric acid, *trans*-ferulic acid, and coumarin were evaluated as well (Fig. 3). Tandem mass spectrometry on the target masses produced diagnostic fragment ion masses for each molecule, and the abundance of these ions was compared with prednisone spiked into the samples. Overall, there were increased amounts of SA in BTH-treated plants (Fig. 4). Meanwhile, the amounts of ACC decreased (Fig. 4). Decreased amounts of ACC agree with increased amounts of ACO, which would deplete the ACC pool to form ET. As for ABA and auxin, there were no overall alterations in their abundance (Fig. 4). There also were no apparent alterations in the amounts of *trans*-cinnamic acid, *p*-coumaric acid, *trans*-ferulic acid, and coumarin (Fig. 4).

DISCUSSION

Rust fungi, because of their wind dispersal and rapid adaptability, continue to cause problems in agriculture; not just

Fig. 3. A phenylpropanoid biosynthesis enzymatic pathway for *Phaseolus vulgaris*. Enzymes with increased accumulation from benzo(1,2,3)thiadiazole-7-carboxylic acid *S*-methyl ester (BTH) treatment are in blue. The pathway was produced by Kyoto Encyclopedia of Genes and Genomes: [EC 4.3.1.24]=Phvul.001G177700, Phvul.008G289500; [EC 1.14.14.91]=Phvul.008G247400; [EC 6.2.1.12]=Phvul.011G084300, Phvul.003G147000; [EC 2.3.1.133]=Phvul.010G162700; [EC 1.14.14.96]=Phvul.001G117900; [EC 3.1.1.-]=Phvul.003G140500; [EC 2.1.1.104]=Phvul.003G047900, Phvul.004G172600; [EC 1.2.1.44]=Phvul.005G182100; [EC 1.2.1.68]=Phvul.002G314000; [EC 1.1.1.195]=Phvul.007G039800; [EC 2.1.1.68]=Phvul.009G258700; [EC 1.1.1.7]=Phvul.007G107400, Phvul.006G075900, Phvul.007G082700, Phvul.008G086800; [EC 2.4.1.111]=Phvul.002G096500; [EC 3.2.1.21]=Phvul.007G250300.

to beans, but to soybeans, wheat, sugarcane, and other crops. The natural immune systems for these plants are strong and, with the aid of R proteins, can activate series of proteins, enzymes, and biochemicals that can thwart rust infection—at least until the rust fungus adapts to overcome the R-protein defense mechanism and renders that plant susceptible to

disease. We show here, however, that topical application of BTH can precondition a susceptible bean plant to be immune to rust (Fig. 1A and B).

BTH application has a drastic effect on the leaf proteome. One observation is that BTH appears to activate many of the same SA defense pathways that are activated by R proteins. For

Fig. 4. Relative accumulation of metabolites in bean leaves treated with benzo(1,2,3)thiadiazole-7-carbothioic acid *S*-methyl ester (+) compared with nontreated controls (-). Units are metabolite peak areas $\times 10^6$ normalized to prednisone peak areas. Peak areas were estimated with Skyline software.

example, both R proteins and BTH-induced immune responses increase the accumulation of EDS1, SAG101, and NDR1, proteins that function in the SA-mediated defense pathway (Century et al. 1997; Cui et al. 2017; Feys et al. 2005). For the most part, these findings were expected, because it is established that BTH induces SAR and that SA is crucial for SAR (Gaffney et al. 1993; Görlach et al. 1996). Nevertheless, we were intrigued by unexpected findings like the increase of so many receptor-like kinases with possible roles in defense activation (Tables 1 and 2). Increased amounts of these proteins could explain a heightened awareness and resistance to a broad spectrum of pathogens that comes from BTH application (Lawton et al. 1996). Increased amounts of these kinases also would explain the sustained increased amounts of phosphoproteins that we observed (Table 1). Interestingly, there were two CIPP1 protein phosphatases that increased, the orthologs of which are commonly induced during SAR in *A. thaliana* (Blanco et al. 2009; Ding et al. 2018; Krinke et al. 2007; Wang et al. 2005). It is likely that these phosphatases work in concert with the kinases to regulate SA-mediated defense responses (Liu et al. 2018).

Not only did BTH application increase protein accumulation, it caused significant protein reductions as well. We observed reduced amounts of chloroplast proteins and reduced amounts of FLA2 that controls auxin responses and reduced amounts of the ABA-regulated water transporter PIP2;7. We previously observed reductions of FLA2 and PIP2;7 in beans with hypersensitive resistance to bean rust (Lee et al. 2009). This further substantiates the evidence that BTH alters defense pathways similarly to R-protein activation. We also previously observed reduced amounts of chloroplast proteins in beans undergoing hypersensitive resistance to rust (Lee et al. 2009). At that time, we attributed the reduction of chloroplast proteins to chlorosis, a common disease symptom arising from a loss of chlorophyll. But now, having observed that BTH reduces the accumulation of many similar chloroplast proteins, we recognize that alterations in the chloroplast proteome may be part of a coordinated defense response. Intriguingly, the chloroplast and ABA alterations may be linked, because ABA is synthesized in the chloroplast and it coordinates a chloroplast retrograde signal to affect nuclear gene expression back to the chloroplast (Zhao et al. 2019).

With respect to protection, topical application of BTH outperformed topical application of SA (Fig. 1B and C). This observation stimulates the thought that BTH application causes cellular responses that are different from SA application. A detailed comparison between the two chemicals is warranted, but the phytotoxicity of SA and the response dosages between BTH and SA would first need to be reconciled before the two chemicals could be compared side-by-side. Nevertheless, the current observations presented here are insightful. Indeed, BTH increased the accumulation of SA (Fig. 4) and increased the abundance of proteins that function through the SA-mediated defense pathway (Table 2). But we cannot yet conclude whether BTH induces novel biosynthesis of SA or whether BTH induced the release of SA from stored forms. There was no increase in PBS3 that conjugates glutamate to isochlorismate for the biosynthesis of SA (Rekhter et al. 2019), but there was an increase in SABP2 that converts stored methyl-SA back to SA (Table 2) (Dempsey and Klessig 2012). Regardless of the mechanism that led to the SA accumulation, the results clearly show that BTH affected SA signaling. But BTH also affected other hormonal defense sectors. For example, decreased amounts of ACC and the concomitant increases of ACO, which converts ACC to ET, and the increased amounts of EIN2, the protein that regulates ET-signaling in the presence of ET (Ju et al. 2012), show that this independent hormonal pathway was

induced by BTH (Table 2; Fig. 4). Meanwhile, increased amounts of IOS1 and RAE1 in BTH-treated beans imply that BTH suppressed ABA-regulated responses, while decreased amounts of FLA2 imply a suppression of auxin regulated responses (Table 2) (Hok et al. 2014; Li et al. 2018; Titapiwatanakun et al. 2009). From this, one might assume that BTH reduced ABA accumulation or auxin accumulation, but we did not observe reductions in ABA or auxin in whole leaf extracts (Fig. 4). It is possible that BTH alters the proteomic regulators for ABA and auxin without resulting in overwhelming concentrations changes like we observed for SA. Notwithstanding, the results reveal that BTH application alters proteins that function through many independent hormonal pathways, leading to an intersection of networks that provide disease resistance.

Our previous research indicates that enzymes that are part of the phenylpropanoid pathway are induced during R protein-mediated immunity to bean rust and that reduced amounts of soybean phenylpropanoid pathway enzymes increase susceptibility to the soybean rust fungus (Cooper et al. 2013; Lee et al. 2009). Hence, our observation that BTH increased 20 enzymes that map to the phenylpropanoid biosynthesis pathway model implied that the treated beans accumulated more phenylpropanoids. Our examination of soluble leaf extracts, however, showed no increased accumulation of pools of *trans*-cinnamic acid, *p*-coumaric acid, *trans*-ferulic acid, and coumarin. There are multiple reasons that explain this observation. First, this type of analysis does not describe flux or flow of these phenylpropanoid intermediates needed to synthesize more complex derivatives and glycosylated conjugates. In other words, there may not be increased pools of these intermediates if flow is accelerated by downstream enzyme activity. Consequently, it would be better to examine the accumulation of end-products (e.g., SA or an end-product substrate like ACC). Because BTH increases lignification in beans (Iriti and Faoro 2003), lignified end-products could be good indicators. But lignin is not easily solubilized and there are many different types of lignin, the masses of which are not easily predictable for our targeted mass spectrometry method. Another reason that may explain unaltered amounts of *trans*-cinnamic acid, *p*-coumaric acid, *trans*-ferulic acid, and coumarin is enzyme homology. While the enzymes we detected are homologous to phenylalanine ammonia lyase that produces *trans*-cinnamic acid, *trans*-cinnamate 4-monooxygenase that produces *p*-coumaric acid, caffeic acid 3-*O*-methyl transferase that catalyzes *trans*-ferulic acid, and beta-glucosidase that produces coumarate in the phenylpropanoid biosynthesis pathway defined by the Kyoto Encyclopedia of Genes and Genomes (KEGG) database, the bean genome encodes at least five other phenylalanine ammonia lyases, two other *trans*-cinnamate 4-monooxygenases, 11 other caffeic acid *O*-methyl transferases, and 26 other beta-glucosidases assigned to the KEGG phenylpropanoid pathway. None of these other isoforms exhibited quantitative proteomic differences. Hence, we must consider the possibility that the enzymes with increased accumulation may synthesize distinct products different from the ones we presumed and targeted. Without deeper knowledge of the array of phenylpropanoid derivatives produced in beans, we must limit ourselves to the general conclusion that the increase of at least 20 enzymes in the phenylpropanoid pathway likely contributed to disease resistance by amplifying the output of this pathway in one form or another. Other studies have reached this general conclusion by showing that some enzymes important for phenylpropanoid biosynthesis and some specific phenylpropanoid derivatives including phytoalexins are critical for rust resistance (Beyer et al. 2019; Cooper et al. 2013; Lygin et al. 2009; Maffi et al. 2011; Pandey et al. 2011).

Overall, our results demonstrate that a 72-h exposure to BTH is sufficient to protect susceptible beans from bean rust fungal infection. BTH protects against bean rust by amplifying SAR and by altering regulators of intersecting hormonal pathways, much like R proteins do. But BTH also increases the accumulation of a variety of receptor-like kinases and other proteins involved in immunity. This protein amplification and sustained phosphorylation likely renders beans hyperaware to a broad spectrum of pathogens, including rust. We encourage others to consider SAR inducers to protect beans against rusts, especially when there is a concern that R proteins are no longer effective.

MATERIALS AND METHODS

Rust fungus and growth conditions.

BTH experiments were performed three separate times in early February, late February, and early March of 2019. Each time, two primary leaves from 10, 7-day-old *P. vulgaris* variety Black Valentine were sprayed with a liquid suspension of Actigard (Syngenta) at 300 mg per liter + 0.002% Tween-20 (0.7 mM BTH). A separate set of plants was sprayed with water + 0.002% Tween-20. Seventy-two hours later, six of the primary leaves were collected and frozen. The remaining 14 primary leaves on 10 plants were sprayed with a liquid suspension of uredospores of *Uromyces appendiculatus* race 41 + 0.025% β -ionone or 0.01% Tween-20, were placed in an 18°C dew chamber for 12 h, and were then moved to indoor growth carts with fluorescent lighting (Lee et al. 2009). At 10 days post-inoculation, rust pustules were counted in multiple 1-cm² leaf areas. This average number was multiplied by leaf area to obtain an estimate of the total number of pustules. A separate set of beans was sprayed with 4.8 mM SA (MP Biomedicals, Solon, OH, U.S.A.) + 0.002% Tween-20 or water + Tween-20 and were challenged as above.

Peptide preparation.

Three grams of leaves per BTH treatment per replicate were frozen in liquid nitrogen and were ground to a powder with a mortar and pestle and were further triturated in 2 volumes of 100 mM Tris, pH 8.5, 10 mM dithiothreitol, 1 mM EDTA, 0.5% dodecyl beta maltoside, plus manufacturer-recommended concentrations of cOmplete proteinase inhibitor (Roche, Mannheim Germany) and PhosSTOP phosphatase inhibitor (Roche). The extract was sonicated for 30 s, and then, it was centrifuged at 1,000 $\times$ g for 10 min. Proteins were precipitated in acetone and 25% trichloroacetic acid and were isolated by centrifugation at 15,000 $\times$ g. The proteins were washed in acetone three times and were dried and resuspended in 8 M urea, 100 mM Tris, pH 8.5, and 1% dodecyl beta maltoside. The protein concentration was determined by bicinchoninic acid assay (Pierce, Rockford, IL, U.S.A.). Proteins (approximately 600 μ g) were reduced in Tris(2-carboxyethyl)phosphine, were carboxyamidomethylated with iodoacetamide, and were digested overnight at 37°C with Poroszyme immobilized trypsin (Thermo Fisher Scientific, Waltham, MA, U.S.A.). The digested peptides were purified by reverse phase chromatography, using Waters Oasis HLB 3 cc Vac Cartridges (Waters Corporation, Milford MA, U.S.A.). Peptides from each sample (360 μ g each) were labeled with TMT 6-plex reagents according to manufacturer instructions (Thermo Fisher Scientific). The samples were dried and resuspended in 0.1% trifluoroacetic acid and peptides were C18-purified and their concentrations were measured with the Pierce quantitative colorimetric peptide assay (Thermo Fisher Scientific). Equivalent quantities of the tagged samples were combined to yield a 1- μ g aliquot that was analyzed by mass spectrometry (below) to

determine label incorporation percentage (>99%) and to estimate quantitative ratios between samples. Based on the quantitative ratios, labeled samples were mixed together. A 600- μ g aliquot was fractionated by high-pH reverse-phase high-pressure liquid chromatography through a Waters Xbridge 3.5 μ m C18 column (4.6 $\times$ 15 cm) with a Dionyx UltiMate 3000 pump controlling a 38-min linear gradient from 4 to 28% acetonitrile and 0.5% triethylamine, pH 10.7 (Wang et al. 2011). Seventy-two fractions were pooled by concatenation and were dried and resuspended in 5% acetonitrile and 0.1% formic acid (Wang et al. 2011). For another 350- μ g aliquot, phosphopeptides were purified with the Pierce high-select-Fe-NTA phosphopeptide enrichment kit. The phosphopeptides, the depleted flow-through, and 3 column washes were reserved, dried, and resuspended in 5% acetonitrile and 0.1% formic acid. Peptide concentrations were measured for 12 pools plus a void volume fraction, the phosphopeptide fraction, the depleted phosphopeptide flow-through fraction, and the combined depleted column washes.

Mass spectrometry.

Peptides (approximately 1 μ g per fraction) were separated on a 75- μ m (inner diameter) fused silica capillary emitter pulled to a 5- μ m tip with a P-2000 Sutter laser puller (Sutter Instrument, Novato, CA, U.S.A.), packed with 22 cm of a 2.5- μ m Synergi Hydro-RP C18 (Phenomenex, Torrance, CA, U.S.A.), and coupled directly to a Dionex UltiMate 3000 RSLC nano system (Thermo Fisher Scientific) controlling a 180-min linear gradient from 3.2 to 40% acetonitrile and 0.1% formic acid at a flow rate of 300 nl per minute. Peptides were electrosprayed at 2.4 kV into an Orbitrap Fusion Lumos Tribrid mass spectrometer (Thermo Fisher Scientific) operating in data-dependent mode with positive polarity and using 445.12003 *m/z* as an internal mass calibrant. Quadrupole isolation was enabled and survey scans were recorded in the Orbitrap at 120,000 resolution over a mass range of 400 to 1,600 *m/z*. The instrument was operated in top speed mode using the Multinotch MS³ method with a cycle time of 3 s (Isasa et al. 2015; McAlister et al. 2014). The automatic gain control (AGC) target was set to 200,000 and the maximum injection time was set to 50 milliseconds (ms). The most abundant precursor ions (intensity threshold 5,000) were fragmented by collision-induced dissociation (35% energy) and fragment ions were detected in the linear ion trap (AGC 10,000, 50 ms maximum injection). Analyzed precursors were dynamically excluded for 45 s. Multiple MS² fragment ions were captured using isolation waveforms with multiple frequency notches and fragmented by high energy collision-induced dissociation (65% normalized collision energy). MS³ spectra were acquired in the Orbitrap (AGC 100,000; maximum injection time 120 ms, 60,000 resolution scanning from 100 to 500 *m/z*). These mass spectrometry data files can be retrieved from the MassIVE database (MSV000084162).

Peptide matching and statistics.

Mass spectrometry data were processed with Proteome Discoverer 2.1 (Thermo Fisher Scientific), which extracted MS² spectra for peptide identification and MS³ spectra for peptide quantitation. MS² spectra were searched with Mascot 2.6.1 against the *P. vulgaris* PI G19833 protein database (27,433 records in the Pvulgaris_442_v2.1.protein_primary-TranscriptOnly.fa file, in the Phytozome database, appended with a list of 172 sequences to detect common contaminants). Search parameters were for tryptic digests with two possible missed cleavages, fixed amino acid modifications for chemically modified cysteine and labeled N-terminal and internal lysine (+57.021 Da, C; +229.163 Da, K), variable oxidized methionine and phosphorylated serine, threonine, and tyrosine

(+15.995 Da, M; +79.966 Da, S, T, and Y), monoisotopic mass values, ± 10 ppm parent ion mass tolerance and ± 0.6 Da fragment ion mass tolerance. Peptide spectrum matches (PSMs) were processed by Percolator, using delta Cn (0.05), strict FDR (0.01), relaxed FDR (0.05), and PEP (0.05) settings. Additional filters limited Mascot ions scores (≥ 13) and PSMs and peptide PEPs (strict 0.01; relaxed 0.05). The estimated PSM strict and relaxed FDR was 0.002. Peptides were assigned to logical protein groups using parsimony. Proteins were quantified on summed S/N ratios for each TMT channel for 61,608 qualified PSMs for unique peptides (isolation interference $< 25\%$; S/N ratio average of all channels > 2 ; at least medium confidence) (Supplementary Dataset S2). The most confident centroid within 0.003 Da of the expected mass of the reporter ions was used. TMT signals also were corrected for isotope impurities (lot-specific data provided by the manufacturer). Missing TMT signal values were replaced with a minimum value of 1 for 98 qualified PSMs. Matches to contaminants and decoys were removed from the dataset, as were proteins with quantitative signal sums across all six channels < 91 or less than two PSMs contributing to the qualified quantitative signal sum. Protein quantitative values for each channel were normalized by the sum of quantitative values for all proteins for each channel and were scaled to 100. An empirical Bayes statistics test was used in the LIMMA package in the R software package to measure significant differences and to limit FDR to ≤ 0.05 (D'Angelo et al. 2017).

Protein sequences were compared with the Swiss-Prot database (downloaded April 30, 2019) with BLASTP. Subcellular localization was predicted with DeepLoc-1.0 (Almagro Armenteros et al. 2017). KEGG identifiers were assigned to *P. vulgaris* reference sequences and were mapped to biochemical and cellular pathways (KEGG database).

Metabolite analysis.

Primary bean leaves sprayed with BTH or with water were sampled 72 h later for metabolite analysis. Half-leaves from two plants totaling 0.5 g comprised a sample. Three to four samples were analyzed per treatment and the experiments were performed four times. Leaves were ground with a pestle in a mortar with 1 ml of ethanol. Prednisone (50 pmol) was added to the slurry. The slurry was transferred to a tube and was centrifuged for 10 min at $12,000 \times g$. The supernatant was passed through an Oasis Prime HLB Plus Light 100-mg cartridge primed with 50% methanol and equilibrated with water. Metabolites were eluted with acetonitrile and were dried in a speed vac. Metabolites were resuspended in 1 ml of 5% acetonitrile and 0.1% formic acid, and 1 μ l was injected into the Orbitrap Fusion for analysis. The settings were the same as above with the following alterations. The Dionex pump controlled a 40-min linear gradient from 3.2 to 40% acetonitrile and 0.1% formic acid at a flow rate of 300 nl per minute. The ion transfer tube temperature was 200°C. Quadrupole isolation was enabled and survey scans were recorded in the Orbitrap at 30,000 resolution over a mass range of 50 to 500 m/z . The AGC target was set to 400,000. A targeted MS² method was performed on the protonated monoisotopic precursors for SA (139.039 m/z), ACC (102.05 m/z), methionine (150.06 m/z), ABA (265.143 m/z), auxin (indole-3-acetic acid, 176.071 m/z), *trans*-cinnamic acid (149.06 m/z), *p*-coumaric acid (165.055 m/z), *trans*-ferulic acid (195.065 m/z), coumarin (147.044 m/z), and prednisone (359.19 m/z). Precursors selected within a 1.6-Da isolation window were fragmented by high-energy collision-induced dissociation (30% normalized collision energy), and fragment ions were resolved in the Orbitrap at 30,000 resolution. Raw result files were analyzed with Skyline version 4.2.0.19009. Peak areas were generated from MS² protonated ion transitions for SA (139.0390, 121.0290, 93.0340

at approximately 18 min), ACC (56.0500 at approximately 5 min), methionine (104.0530, 102.0560, 56.0500 at approximately 6 min), ABA (201.1270, 187.1110, 173.1330, 163.0760, 135.0810, 121.0650 at approximately 17 min), auxin (130.0650, 117.0570, 103.0540, 77.0390 at approximately 30 min), *trans*-cinnamic acid (131.0500, 103.0550, 77.0391 at approximately 12 min), *p*-coumaric acid (147.0450, 119.0450, 91.0540 at approximately 26 min), *trans*-ferulic acid (177.0550, 149.0600, 145.0285, 117.0340 at approximately 27 min), coumarin (147.0446, 119.0490, 91.0550, 77.0391 at approximately 26 min), and prednisone (147.0810, 135.0810, 121.0650, 95.0870 at approximately 35 min). Because in-source fragmentation of methionine generates a 56.0500 m/z ion that is an isomer of the ACC transition, peak area selections for ACC were terminated when the first transitions for methionine were detected. Metabolite peak areas were normalized to prednisone peak areas per sample. The transitions originally were selected by analyzing MS² spectra from 500 pmol of each purified metabolite (purchased from Sigma-Aldrich, St. Louis). Transition masses are supported by predicted molecular derivative structures at PubChem.

AUTHOR-RECOMMENDED INTERNET RESOURCES

KEGG database: <https://www.kegg.jp>

MassIVE database: <https://massive.ucsd.edu/ProteoSAFe/static/massive.jsp>

Phytozome database: <https://phytozome.jgi.doe.gov>

PubChem: pubchem.ncbi.nlm.nih.gov

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