Brassinosteroid Enhances Resistance to Fusarium Diseases of Barley

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ABSTRACT

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Fusarium pathogens are among the most damaging pathogens of cereals. These pathogens have the ability to attack the roots, seedlings, and flowering heads of barley and wheat plants with disease, resulting in yield loss and head blight disease and also resulting in the contamination of grain with mycotoxins harmful to human and animal health. There is increasing evidence that brassinosteroid (BR) hormones play an important role in plant defense against both biotic and abiotic stress agents and this study set out to determine if and how BR might affect Fusarium diseases of barley. Application of the epibrassinolide (epiBL) to heads of ‘Lux’ barley reduced the severity of Fusarium head blight (FHB) caused

by *Fusarium culmorum* by 86% and reduced the FHB-associated loss in grain weight by 33%. Growth of plants in soil amended with epiBL resulted in a 28 and 35% reduction in Fusarium seedling blight (FSB) symptoms on the Lux and ‘Akashinriki’ barley, respectively. Microarray analysis was used to determine whether growth in epiBL-amended soil changed the transcriptional profile in stem base tissue during the early stages of FSB development. At 24 and 48 h post *F. culmorum* inoculation, there were 146 epiBL-responsive transcripts, the majority being from the 48-h time point ($n = 118$). Real-time reverse-transcription polymerase chain reaction analysis validated the results for eight transcripts, including five defense genes. The results of gene expression studies show that chromatin remodeling, hormonal signaling, photosynthesis, and pathogenesis-related genes are activated in plants as a result of growth in epiBL.

Over the course of evolution, plants have developed complex relationships with microbes, and microbial pathogens have evolved the ability to cause disease on a range of plants. The extent of plant defense responses is influenced by plant hormones. The main plant hormones involved in plant–pathogen interactions are salicylic acid (SA), jasmonic acid (JA), ethylene (ET), and brassinosteroid (BR). The study of BR signaling and its role in disease responses is in its infancy, relative to SA, JA, and ET. BRs and BR signaling have complex positive and negative roles in innate immune responses (49). BR is perceived by the receptor BR-insensitive 1 (BRI1); BRI1 competes with the immune response for the membrane receptor BAK1 (22) but BR can also induce immunity in a BAK1-independent manner (1,7).

Nakashita et al. (35) were the first to report that BR treatment enhanced biotroph and hemibiotroph resistance. They demonstrated that the BR hormone brassinolide can function in resistance to a broad spectrum of pathogens in tobacco and rice. Although the mechanisms by which BR induced resistance in tobacco and rice are unknown, BR signaling was shown to positively regulate antioxidant systems and metabolites that protect cells from stress (5). BR signaling can induce expression of the gene encoding nicotinamide adenine dinucleotide phosphate

oxidase to regulate H_2O_2 production; the regulation of oxidative stress is a major component of both abiotic and biotic stress tolerance. Active BR signaling can also upregulate nitric oxide production, which promotes abscisic acid (ABA) biosynthesis, resulting in stress tolerance. The transcription factors BZR1 and BES1 play a central role in BR-regulated plant responses to disease. BES1 induces and binds to a key defense regulator, AtMYB30, and together they function cooperatively to promote BR target gene expression (29). BZR1 is a negative regulator of biotic response genes, including *FLS2* and *SNC1*, that are directly involved in defense against pathogens. BR-mediated stress tolerance in *Arabidopsis* shows interactions with ABA, ET, and SA pathways (17), and BZR1 was shown to directly regulated genes involved in many hormone signaling or biosynthesis pathway (45). BR is implicated in mediating abiotic stress responses through NPR1, a master regulator of SA defense signaling (17,18). However, BR treatment induces pathogenesis-regulated 1 (*PR1*) expression in an NPR1-independent manner (17).

In all, ≈ 70 BRs have been isolated from plants (6). Epibrassinolide (epiBL) is a major end product of the BR biosynthesis pathway and is present in almost every plant tissue or organ. It was shown to promote stem elongation and cell division (20,33). The potential of epiBL as a protectant against both biotic and abiotic stress damage has been reported in various agricultural crops (3,19,48). Here, we demonstrate that BR has the potential to protect barley against Fusarium diseases, including the economically devastating Fusarium head blight (FHB) disease, under glasshouse conditions. Using seedling studies, we assessed how epiBL application influences early plant transcriptomic responses to the pathogen *Fusarium culmorum*. Based on the results, we

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discuss the metabolic changes underpinning BR-mediated control of Fusarium diseases of barley.

MATERIALS AND METHODS

Plants, fungal, and chemical materials. The two-row barley (*Hordeum vulgare*) 'Lux' was kindly supplied by Powerseeds, Kildare, Ireland. 'Akashinriki' was kindly provided by K. Sato, Barley Germplasm Centre, Okayama University, Japan. Heads and seedlings of these two genotypes are susceptible to *Fusarium* fungi (24). *F. culmorum* is one of the most common species that cause FHB disease (42); the *F. culmorum* isolates used in this study were strain FCF 200 (kindly supplied by Paul Nicholson, John Innes Centre, Norwich, UK). The fungus was stored at -70°C and, prior to use, were subcultured onto potato dextrose agar (PDA) (Difco, UK) plates and incubated at 25°C for 5 days. Fungal conidial inoculum was produced in mung bean broth as described by Brennan et al. (9). epiBL was purchased from Sigma-Aldrich (St. Louis) and a 20-mM stock was prepared in ethanol and subsequently diluted in sterile distilled water to the required concentration.

FHB experiments. All head blight experiments were conducted in a glasshouse, as previously described (24). Barley Lux plants were grown in a heated glasshouse (22°C , 16-h day, and 12°C , 8-h night; maximum temperature of 27°C). At midanthesis, heads (two per plant) were sprayed to runoff with either 20 μM epiBL or water (control plants). At 24 h post hormone application, the same heads were treated with either Tween 20 or *F. culmorum* conidia (10^5 ml^{-1}), as previously described (24). Visual disease symptoms were recorded at growth stage (GS) 80 (start of dough development) based on the percentage of bleached spikelets per head. Treated heads were harvested at GS 99 (50). The number of seed per head and total seed yield (in grams) per head were recorded. Each treatment combination was applied to 16 plants (two heads per plant) and the experiment was conducted twice (February to April 2012 and March to May 2012) in independent glasshouses in a randomized layout.

Seedling blight experiments. Fusarium seedling blight (FSB) experiments were conducted as described by Khan et al. (25). The plants were grown in a climate-controlled growth room with day and night temperatures of 20 and 12°C , respectively, with a 12-h light period ($700\text{ }\mu\text{mol m}^{-2}\text{ s}^{-1}$) and constant humidity of 85%. Seed of Lux and Akashinriki barley were germinated and grown in a 6-cm-diameter pot containing soil amended with 10 ml of either water or 20 μM epiBL, and the stem bases of 10-day-old seedlings were treated with either 0.2% Tween 20 in 1% (wt vol $^{-1}$) agar (Difco Laboratories, Detroit) or conidia ($1 \times 10^6\text{ ml}^{-1}$) of *F. culmorum*. The stem base samples were harvested at 15 days post fungal treatment. Seedling blight stem base disease symptoms were scored as product of lesion length (in centimeters) by lesion color (lesion color scale: 0, no disease; 1, very slight brown necrosis; 2, slight to moderate brown necrosis; 3, extensive brown necrosis; and 4, extensive black necrosis) (36). This experiment was conducted thrice, and each time it included three replicate pots (each containing two plants) per treatment combination, arranged in a randomized layout. Similar experiments were conducted in order to analyze gene expression via either microarray analysis or reverse-transcription polymerase chain reaction (RT-PCR) in Akashinriki. For microarray analysis, three replica experiments were conducted, each including two treatments (treatment 1 involved growth in epiBL-amended soil and stem inoculation with *F. culmorum* while treatment 2 involved growth in water-amended soil and stem inoculation with *F. culmorum*). Three experiments conducted for RT-PCR studies involved four treatments (growth in soil amended with water or epiBL and stem treatment with either Tween20 and agar or *F. culmorum* and agar). In all gene expression experiments, samples were harvested at either 24 or 48 h post stem base treatment, flash frozen in liquid nitrogen, and

stored at -70°C prior to RNA extraction, and each experiment included six replicate pots (each containing two plants) per treatment combination.

In vitro plate assay. PDA was amended with epiBL (0, 0.2, 2, 10, 20, 50, 100, or 200 μM). Plates were inoculated with a 5-mm-diameter plug of *F. culmorum*, which was harvested from a 7-day-old PDA plate. Plates were incubated at 25°C in darkness. After 72 h, the colony diameter (in centimeters) was measured. The experiment was carried out twice, each containing three technical replicates per treatment.

RNA extraction. Total RNA was extracted from the stem base samples using the protocol described by Chang et al. (10), DNaseI treated according to the manufacturer's instructions (Invitrogen Corp., Carlsbad, CA), and resuspended in diethyl pyrocarbonate-treated water. The quantity of RNA in samples was assessed using an Eppendorf Biophotometer (Eppendorf AG, Hamburg, Germany) according to the manufacturer's instructions. RNA quality of samples was assessed by estimating the RNA integrity number, which averaged >8 , indicating high-quality RNA.

Microarray analysis. Microarray analysis was used to determine the effect of growth in epiBL-amended soil (relative to mock treatment) on the gene expression in *F. culmorum*-inoculated stem bases of Akashinriki at either 24 or 48 h post fungal inoculation (i.e., comparison of gene expression in plants treated with fungus versus plants treated with epiBL plus fungus). Microarray production, hybridization, and data analysis were performed following the minimum information about a microarray experiment (MIAME) guidelines for international standardization and quality control of microarray experiments (8). Microarray analysis was conducted using three composite samples per treatment; composite samples were produced by pooling equal amount of the total RNA from the five replicate samples per treatment per experiment per time point. Total RNA (1 μg) from each sample was converted to double-stranded cDNA with the Bioarray single-round RNA amplification and labeling kit (Enzo Life Sciences, PA). After second-strand synthesis, the cDNA was purified with the cDNA purification kit (Enzo Life Sciences). The resulting double-stranded DNA was then used to generate multiple copies of biotinylated cRNA by in vitro transcription with the Bioarray Highyield RNA transcript labeling kit (Enzo Life Sciences). The ratio of absorbance at 260/280 nm and the yield of each of the cRNAs were determined and the quality of these samples was assessed using an Agilent bioanalyzer (Agilent Technologies, Palo Alto, CA). Biotinylated cRNA (10 μg) spiked with *biob*, *bioc*, *biod*, and *cre* (hybridization controls) was hybridized to an Affymetrix barley genome microarray for 16 h at 45°C . Following hybridization, all arrays were washed and stained in an Affymetrix Genechip fluidics station. Stained arrays were scanned with an Affymetrix Genechip scanner 3000. Quality checks and data analyses were carried out using Affymetrix Genechip operating software (gcoss) and quality reporter. The array data was normalized per chip and per gene. The per-chip normalization was carried out to the median. For per-gene normalization, the expression value obtained for each gene on each of the three "disease-treated" arrays was divided by the mean value obtained for that gene in the three control-treated arrays. Finally, two lists of significant probes (epiBL-responsive at either 24 or 48 h post fungal treatment) were generated using a student *t* test with a fold-change threshold of ≥ 2 and ≤ -2 , with a *P* value < 0.05 (the cut-off previously used) (15). Raw data are in the process of being deposited in MiamExpress.

Sequence analysis. For each probe set, annotations of associated genes or gene homologs were obtained directly from the Affymetrix website (<https://www.affymetrix.com/analysis/netaffx/showresults.affx>) or by BLASTx analysis against the nonredundant protein database (2) using the National Centre for Biotechnology Information blast resource (www.ncbi.nlm.nih.gov). The cut-off value of 10^{-15} was used as a threshold for the

expectation scores (e values), and only homologies with an e value of less than the threshold were regarded as significant. Whenever the two descriptions disagreed, the BLASTx description was selected. Based on putative protein functions, genes were assigned to different functional categories according to the Munich Information Centre for Protein Sequences (MIPS) Functional Catalogue scheme (release 2.1) (http://mips.helmholtz-muenchen.de/proj/funccatDB/search_main_frame.html) (32).

Real-time RT-PCR analysis. Real-time RT-PCR was used to analyze the temporal expression of select epiBL-responsive transcripts of relevance to plant defense. RT of total RNA was conducted as described by Ansari et al. (4), except that the primer used was oligo dT₁₂₋₁₈ (Invitrogen). RT products were diluted to 200 µl, and 2.5 µl was PCR amplified in a 25-µl volume reaction containing 12.5 µl of Premix Ex Taq (Perfect Real Time; Takara, Japan) and 100 nM each forward and reverse transcript-specific primers (Table 1). PCR reactions were conducted in a Stratagene Mx3000 real-time PCR machine (Stratagene) and the program consisted of 1 cycle of 95°C for 10 s, 40 cycles of 95°C for 5 s and 60°C for 30 s, and 1 cycle of 95°C for 60 s. Data were analyzed using Stratagene Mx3000 software (Stratagene). The housekeeping gene used for normalization of real-time RT-PCR data was α -tubulin (Affymetrix Contig127_s_at); real-time quantification of target gene and of the housekeeping gene was performed in separate reactions. The threshold cycle (Ct) values obtained by real-time RT-PCR were used to calculate the accumulation of target gene (relative mRNA accumulation) relative to α -tubulin transcript by the $2^{-\Delta\Delta Ct}$ method, where $\Delta\Delta Ct = (Ct \text{ target gene} - Ct \alpha\text{-tubulin})$ (31). Results were based on the average obtained for at least two replicate RT-PCR reactions per sample.

Statistical analysis. Real-time RT-PCR data for Contig10686_at, Contig6276_s_at, and Contig2170_at were normally distributed, as determined using the Ryan Joiner test within Minitab (Minitab release 13.32, 2000; Minitab Inc.). All other data sets were non-normally distributed. All data sets except the percentage of infected spikelets and seed weight per head data of the FHB experiment could not be transformed to fit a normal distribution using the Johnson transformation within Minitab (Minitab release 13.32, 2000; Minitab Inc.). The homogeneity of data sets across replicate experiments was confirmed by two-tailed correlation analysis (non-normal data: Spearman Rank; normal data: Pearson product moment) conducted within the Statistical Package for the Social Sciences (SPSS 11.0; SPSS Inc.) ($r \geq 0.798$; $P = 0.01$). Therefore, data sets from the replicate experiments were pooled for the purposes of further statistical analysis. The significance of treatment effects was analyzed within Statistical Package for the Social Sciences (SPSS 11.0, SPSS Inc.) by either (i) normally distributed data (one-way analysis of variance with post hoc pairwise least significance difference comparisons, $P = 0.05$), or (ii) non-normally distributed data (the Kruskal-Wallis H test).

RESULTS

Application of epiBL reduced the severity of FHB disease of barley. Glasshouse experiments were conducted in order to determine the effect of epiBL on FHB disease of barley (Lux). In the absence of BR, inoculation with the pathogen *F. culmorum* resulted in 31% of spikelets displaying typical FHB bleaching symptoms by GS 80 ($P < 0.01$) (Fig. 1A and C). However, when epiBL was applied 24 h prior to pathogen inoculation, only 4% of spikelets showed symptoms (Fig. 1C). EpiBL also reduced losses in grain number and weight caused by FHB disease. Plants pretreated with epiBL prior to *Fusarium* spp. contained an average of 3.6 more grain per head and an increase of 0.21 g in grain weight per head (Fig. 1D and E) compared with those only treated with the fungus. Thus, epiBL application reduced the FHB-associated loss in grain weight by 33% ($P < 0.01$). Grains from epiBL + fungus-treated heads were visually similar to those obtained from control plants (non-pathogen treated); in general, they did not display the fungal growth and shrinkage evident in grains treated with pathogen (no epiBL treatment) (Fig. 1B).

Soil amendment with epiBL reduced the severity of FSB disease of barley. Seedling assays were conducted to determine the effect of soil amendment with epiBL on the development of FSB symptoms on *F. culmorum*-inoculated stem base tissue of Lux and Akashinriki. It resulted in a 28 and 35% reduction, respectively, in the severity of visual disease symptoms on Lux and Akashinriki relative to the level of disease symptoms observed on *F. culmorum*-inoculated plants grown in soil without epiBL amendment ($P < 0.05$; Fig. 2).

No evidence that epiBL inhibits the growth of *Fusarium* spp. in vitro. In vitro plate assays were carried out to determine whether there was any direct inhibitory effect of epiBL on fungal growth on PDA. Even at the highest concentration tested (200 µM), epiBL did not inhibit the radial growth of the pathogen (Supplemental Figure 1).

Growth in epiBL-amended soil systemically alters the regulation of defense transcripts. Microarray analyses were used to determine the effect of growth in epiBL-amended soil on the transcriptome profile in *Fusarium*-infected seedling tissue, relative to the transcriptome of *Fusarium*-treated plants grown in nonamended soil. Two treatments were compared: epiBL + pathogen versus no hormone + pathogen. Microarray analyses indicated that, at 24 and 48 h post *F. culmorum* inoculation, 146 transcripts were differentially regulated (≥ 2 - and ≤ -2 -fold change) in *Fusarium*-inoculated barley as a result of soil amendment with epiBL (Supplemental Tables 1 and 2). The majority of these were from 48 h, at which time 96 transcripts were upregulated or activated and 22 transcripts were downregulated in stem base tissue of barley plants grown in epiBL-amended soil, compared with the control treatment. At 24 h, 16 were upregulated and 12 transcripts downregulated due to epiBL treatment. Only one

TABLE 1. Affymetrix probes-specific primers used for real time reverse-transcription polymerase chain reaction (RT-PCR) analysis^a

Affymetrix Probe ID ^b	Probe annotation ^c	Forward primer (5'–3')	Reverse primer (5'–3')
Contig5311_at	Caffeic acid O-methyltransferase	GCAGGGGTAACTGTGTGG	GCAGGCTCCAGTAGAAGTG
Contig7886_at	Putative jasmonate-induced protein	GACAGAAGCACAAATGCTGGA	ATTCTTCTGCACGGCTATGG
Contig10686_at	Athogenesis-related group 5 protein	CCAAGGCGTATTCGTATGCT	ATCCAACGACAGCAAGAACC
Contig11534_at	Root-specific lectin precursor protein	CAATATGACGTGCAGGTTGC	TTCGTGCAAGACTACGGTTG
Contig15164_at	Cytochrome P450	ATGCCGATATTCAGCTCAC	GCATGAGCATGAGCAGTAGC
Contig6276_s_at	Abscicic acid-induced protein	CAATTGGAAGGCCATGAGAC	CATGAACGGAAGCAAGGATT
Contig2170_at	Pathogen-induced protein WIR1A	CGTCCGTGCAGAGCATATAA	TTTTTCTCACGCCAGACAT
Contig639_at	Pathogenesis-related protein 4	CCAAGCGTGTACATGGTGTC	CCATTCAAGCTTGCTGCATA
Contig127_s_at	α -Tubulin	GCATGGAGGAGGAGAGTTC	CCAGGAGGCAGGCTTCTAGT

^a Primers were designed using the Primer3 software (version 0.4.0; <http://frodo.wi.mit.edu/primer3/>). Primer specificity was confirmed by melting curve analyses of real-time RT-PCR products (81 cycles of 55°C for 30 s).

^b Affymetrix Probes ID used in the microarray analysis were obtained from PLEX data base (<http://www.plexdb.org>) and the sequence was used for design of primers.

^c Probe annotation as described in Supplemental Tables 1 and 2.

epiBL-responsive transcript encoding a histone H4 (Contig37_at) was common to both time points.

Differentially expressed genes were categorized based on the MIPS functional catalogue (Fig. 3). At 24 h post *F. culmorum*

inoculation, 6 of the 28 transcripts that were downregulated in response to epiBL treatment were histones associated with cell cycle and DNA processing (Fig. 3A). Other notable results at 24 h were the epiBL-mediated upregulation of four defense genes

Fig. 1. Effect of exogenous application of epiBL on the symptoms, grain number, and grain weight (per head) in *Fusarium* head blight (FHB) trials. Plants were treated with either 20 μ M epiBL or water 24 h pretreatment with either *Fusarium culmorum* conidia in Tween20 solution or Tween20 solution. Disease was assessed at growth stage (GS) 80 and plants were harvested at GS 90. **A**, Visualization of the FHB disease symptoms at GS 80. **B**, Grain quality was visually assessed at GS 90. **C**, Disease symptoms at GS 80 were quantified as the percentage of infected spikelets per head. At harvest (GS90), the **D**, number of grains per head and **E**, weight of the grain in each head were determined. Bar indicates standard error of mean. Values followed by * are significantly different at $P \leq 0.01$.

Fig. 2. Effect of soil amendment with epiBL on the development of *Fusarium* seedling blight (FSB) symptoms on the stem base tissue of barley. **A**, 'Lux' and **B**, 'Akashinriki' barley plants were grown in soil amended with either 20 μ M epiBL or water and, after 10 days, stem bases were treated with either Tween 20 in 1% agar or *Fusarium culmorum* conidia in Tween 20 and 1% agar. Fifteen days post stem base treatment, the visual disease symptoms were assessed. FSB symptoms were scored as product of lesion length (in centimeters) by lesion color (lesion color scale: 0, no disease; 1, very slight brown necrosis; 2, slight to moderate brown necrosis; 3, extensive brown necrosis; and 4, extensive black necrosis) (38). Bars indicate standard error of mean least significant difference at $P \leq 0.05$: A = 1.3 and B = 1.9).

(encoding PR4, WIRIA, asparaginase, and xylanase proteins) and four genes involved in glucan metabolism. Seven epiBL-regulated genes at 24 h were of unknown function.

By 48 h, there was a dramatic increase in the number of defense genes responsive to epiBL ($n = 17$), with the majority ($n = 14$) being upregulated due to the hormone treatment of soil. These represented a diverse array of proteins (Fig. 3B), including cytochrome P450s, horcolin, subtilisin, a glutathione-S-transferase, and those responsive to ABA, auxin, and cold. Transcripts encoding a lectin and a jasmonate-inducible protein were downregulated at 48 h due to epiBL treatment. Among the 14 energy-related transcripts differentially regulated in response to epiBL, 7 encode photosystem proteins.

Real-time RT-PCR analysis was performed in order to validate the microarray results for eight defense genes (i.e., effect of epiBL on transcription) and to go one step further (i.e., determine the effect of the pathogen on the transcription of these genes). According to microarray analyses, these genes were either upregulated in response to epiBL (Fig. 4A to D), not greatly (<two-fold) affected by epiBL (PR-5; Fig. 4E), or downregulated due to epiBL (Fig. 4F to H). In all cases, the RT-PCR results concurred with those of microarray analysis in terms of the effect of epiBL on transcript accumulation. Experiments for RT-PCR included treatment additional to those assessed by microarray analysis; that is, (i) no epiBL, no fungus and (ii) no epiBL, fungus. This enabled us to determine that three of the four epiBL-upregulated genes studied were also upregulated by *Fusarium* spp.; these were the cytochrome P450, the pathogenesis protein PR-4, and the pathogen-induced protein WIR1A (Fig. 4A, C, and E; $P < 0.05$). The effects of both *Fusarium* spp. and epiBL on the transcription of these genes was additive. The epiBL-upregulated ABA-induced protein 7 gene was not responsive to *Fusarium* spp. (Fig. 4B). Although the pathogenesis gene PR-5 was responsive to *Fusarium* spp., RT-PCR concurred with microarray results in that epiBL did not greatly affect the transcription of this gene. RT-PCR indicated that caffeic acid O-methyltransferase, root-specific lectin precursor protein, and putative jasmonate-induced protein were all upregulated by *Fusarium* spp. but, as with microarray, were downregulated by 48 h post pathogen treatment due to growth in epiBL-amended soil (Fig. 4F, G, and H).

DISCUSSION

This work demonstrates the potential of epiBL as a control agent for *Fusarium* diseases of barley. FHB disease is a particularly good target for agents such as epiBL due to the narrow infection window, with the optimal infection time being mid-anthesis. The glasshouse performance of control agents will undoubtedly be better than field performance. Others have shown that field application of lower concentrations of BR to barley plants (in doses of ≈ 5 to 15 mg ha^{-1}) significantly decreased the extent of leaf diseases induced by mixed fungal infection and increased the crop yield (39). BR also enhanced cucumber resistance to mildew under field conditions. Cucumber seed treatment with epiBL at $0.1 \text{ mg liter}^{-1}$ followed by foliar application at 25 mg ha^{-1} during flowering stage significantly suppressed mildew (12). Application of BL (in concentrations similar to or higher than used in our study) enhanced resistance of tobacco against *Tobacco mosaic virus*, *Pseudomonas syringae* pv. *tabaci*, and *Oidium* spp. and of rice against *Magnaporthe grisea* and *Xanthomonas oryzae* pv. *oryzae* (35). Effects of BR on diseases may be concentration dependent, as shown by Nakashita et al. (23). Korableva et al. (26) also showed that the effect of BR on disease can be dependent on the method and time of application, as was the case for late blight of potato, where effects varied from negative to positive. BRs were shown to enhance blight by stimulation of mycelial growth and spore formation by *Phytophthora infestans*, and retarding wound repair (47). In contrast, BL application after harvesting prolonged dormancy of potato and enhanced resistance to *P. infestans* infection. We found no evidence that BL affected the mycelial growth of *F. culmorum* under the in vitro fungal culture conditions tested herein.

BRs are involved in plant cell elongation, vascular development, senescence, photomorphogenesis, flowering control, and stress responses (13,27,28). Though it is certain that BRs effect the gene expression of various plant growth and development processes, we still do not have sufficient knowledge about their functional mechanisms. Known BR pathway genes were not highlighted within our microarray study but this is not surprising because the experiment was not designed to elucidate the early responses of plants to epiBL. One of the most striking results

Fig. 3. Effect of barley growth in epi brassinolide (epiBL) on the transcriptional activity in *Fusarium*-infected stem tissue compared with growth in soil amended with water. Plants were grown in soil amended with either water or $20 \mu\text{M}$ epiBL. After 10 days, stem bases were treated with *Fusarium culmorum* conidia in Tween 20 and 1% agar. Microarray analysis was conducted using tissue harvested A, 24 and B, 48 h post pathogen inoculation. RNA was extracted from stem base tissue and results are based on independent microarray analysis for three replica experiments and represent transcripts significantly affected by epiBL treatment (≥ 2 and ≤ -2 -fold change in expression; $P \leq 0.05$). Genes were assigned to functional categories using the Munich Information Center for Protein Sequence Functional Catalogue scheme (release 2.1) (http://mips.helmholtz-muenchen.de/proj/funecatDB/search_main_frame.html).

from the microarray study was the revelation that large numbers of histones were downregulated in *Fusarium*-inoculated tissue as a result of growth in epiBL-amended soil. Müssig et al. (34) found that, although BR did not significantly alter histone gene expression in wild-type *Arabidopsis* plants, it inconsistently downregulated *His1-3* in Br-deficient *dwl-6* mutant plants. This suggests that chromatin remodeling is either an effector of or

affected by epiBL signaling. This adds further weight to the evidence that BR function requires chromatin remodeling (43). Chromatin remodeling plays an important role in SA-mediated systemic acquired resistance (46) and SA has recently been shown to play a role in FHB resistance (16). Chromatin remodeling could be instrumental for the priming of epiBL-responsive loci to respond to subsequent *Fusarium* spp. attack.

Fig. 4. Effect of epibrassinolide (epiBL) amendment of soil on the expression of specific genes in *Fusarium*-infected seedlings, as determined by real-time reverse-transcription polymerase chain reaction analysis. Plants were grown in soil amended with either water or 20 μ M epiBL. After 10 days, stem bases were treated with *Fusarium culmorum* conidia in Tween 20 and 1% agar. RNA was extracted from stem bases tissue harvested at either 24 or 48 h post fungal inoculation. Gene accumulations were quantified relative to that of the housekeeping gene α -tubulin (Contig127_s_at). Gene represented are **A**, cytochrome P450 (*CYP*) (Contig15164_at); **B**, abscisic acid (ABA)-induced protein 7 (*ABA7*) (Contig6276_s_at); **C**, pathogenesis-related protein 4 (*PR-4*) (Contig639_at); **D**, pathogen-induced protein *WIR1A* (Contig2170_at); **E**, PR group 5 protein (*PR-5*) (Contig10686_at); **F**, caffeic acid O-methyltransferase (*COMT*) (Contig5311_at); **G**, root-specific lectin precursor protein (*RSLP*) (Contig11534_at); and **H**, putative jasmonate-induced protein (*JIP*) (Contig7886_at). Genes were identified using Affymetrix barley genome microarray analysis. Results are based on two experiments, each with three replicates per treatment. Bars indicate standard error of mean least significant difference at $P \leq 0.01$: A = 0.027, B = 0.018, C = 0.051, D = 0.027, E = 0.014, F = 0.0001, G = 0.0002, and H = 0.0003).

From the present study, it is clear that BR stimulates select barley defense genes during *Fusarium* infection. It could be that a subset of these genes are primed by the hormone to respond to the pathogen; however, the microarray analysis conducted herein was not extensive enough to discriminate between hormone-responsive and hormone-primed genes. Nevertheless, it did identify interesting defense genes activated by epiBL. The pathogen-induced protein WIR1A protein is involved in cell wall defense of wheat against rust (14). β -1,3-Glucanases were upregulated due to BR treatment in this study, and these have been shown to be antifungal, probably by hydrolyzing their substrates that are structural components in the fungal cell wall (44). Roth et al. (41) also observed that application of BR enhances expression of β -1,3-glucanase, chitinase, and peroxidase in potato tubers. In the present study, it was observed that BR induces the transcription of genes encoding subtilisin and a glutathione-S-transferase safener binding protein 1 which may, in turn, trigger the PR proteins (23,40). Our study also demonstrated that BR induces another class of defense proteins called horcolin that can interact with carbohydrates and trigger a stress-signaling pathway (21,38). Upregulation of ABA and auxin-inducible genes and down-regulation of a jasmonate-inducible gene indicates a complete alteration of the hormonal profile in barley seedling during *Fusarium* infection in response to BR treatment. Recent findings have also made it clear that BR signaling components mainly interact with the signaling elements of other hormonal pathways at the transcriptional level (11). Korableva et al. (26) observed that, under the influence of BRs, ET synthesis in tuber tissues increased. Similarly, epiBL treatment may also trigger ET synthesis in barley seedlings, resulting in upregulation of senescence-associated genes. Thus, our findings further strengthen the concept of crosstalk between BRs and other plant hormones. In a recent study, we observed that indole acetic acid application triggers defense against FHB in barley (37). Another gene worth investigating is steroleosin, which was upregulated in barley seedlings as a result of growth in epiBL during *Fusarium* infection. Because it is involved in plant signal transduction regulated by various sterols (30), its role in BR-mediated signaling will be interesting to study.

In conclusion, the present study confirmed that BR exerts a wide variety of effects on both plant growth and development in diseased plants, from the activation of plant photosystems to chromatin remodeling. Most importantly, it has shown that BR application in barley triggers systemic signals against the plant-pathogenic fungus *F. culmorum*, resulting in a significant reduction in fungal infection and components that contribute to yield loss.

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