A Comparative Transcriptomic and Proteomic Analysis of Hexaploid Wheat's Responses to Colonization by *Bacillus velezensis* and *Gaeumannomyces graminis*, Both Separately and Combined

Xingxing Kang,¹ Lanhua Wang,¹ Yu Guo,¹ Muhammad Zain ul Arifeen,¹ Xunchao Cai,¹ Yarong Xue,^{1,†} Yuanqin Bu,² Gang Wang,³ and Changhong Liu^{1,†}

¹ State Key Laboratory of Pharmaceutical Biotechnology, School of Life Sciences, Nanjing University, Nanjing, China

² Nanjing Institute of Environmental Sciences, Key Laboratory of Pesticide Environmental Assessment and Pollution Control, Ministry of Ecology and Environment, Nanjing, China

³ State Key Laboratory of Cotton Biology, School of Life Sciences, Henan University, Kaifeng, Henan, China

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Tritrophic interactions involving a biocontrol agent, a pathogen, and a plant have been analyzed predominantly from the perspective of the biocontrol agent. To explore the adaptive strategies of wheat in response to beneficial, pathogenic, and combined microorganisms, we performed the first comprehensive transcriptomic, proteomic, and biochemical analysis in wheat roots after exposure to *Bacillus velezensis* CC09, *Gaeumannomyces graminis* var. *tritici*, and their combined colonization, respectively. The transcriptional or translational programming of wheat roots inoculated with beneficial *B. velezensis* showed mild alterations compared with that of pathogenic *G. graminis* var. *tritici*. However, the combination of *B. velezensis* and *G. graminis* var. *tritici* activated a larger transcriptional or translational program than for each single microorganism, although the gene expression pattern was similar to that of individual infection by *G. graminis* var. *tritici*, suggesting a prioritization of defense against *G. graminis* var. *tritici* infection. Surprisingly, pathogen-associated molecular pattern-triggered immunity and effector-triggered immunity made wheat pretreated with *B. velezensis* more sensitive to subsequent *G. graminis* var. *tritici* infection. Additionally, *B. velezensis* triggered a salicylic acid (SA)-dependent mode of induced systemic resistance that resembles pathogen-induced systemic acquired resistance. Wheat plants mainly depend on SA-mediated resistance, and not that mediated by jasmonic acid (JA), against the necrotrophic pathogen *G. graminis* var. *tritici*. Moreover, SA–JA interactions resulted in antagonistic effects regardless of the type of microorganisms in wheat. Further enhancement of SA-dependent defense responses such as lignification to the combined infection was shown to reduce

the level of induced JA-dependent defense against subsequent infection with *G. graminis* var. *tritici*. Altogether, our results demonstrate how the hexaploid monocot wheat responds to beneficial or pathogenic microorganisms and prolongs the onset of take-all disease through modulation of cell reprogramming and signaling events.

Keywords: bacterium–plant symbiosis, biocontrol bacteria, fungus–plant interactions, genomics, induced systemic resistance, metabolomics, molecular signaling, omics, plant defense system, plant disease, plant–microbial interaction, phytopathology, proteomics

Wheat is the second most important food crop globally, serving as a staple food for much of the world's population (Singh et al. 2011; Singh et al. 2018). Global wheat production is severely affected by “take-all”, a major fungal disease caused by *Gaeumannomyces graminis* var. *tritici*, which can cause significant yield and economic losses and remains challenging to control (Bithell et al. 2016). *G. graminis* var. *tritici* is a necrotrophic soilborne pathogen that infects wheat roots via hyphae by penetrating root cortical cells and progressing into the stem, where it mainly disrupts water flow, thus causing stunted growth and premature death of the plant. This fungal disease is not restricted to wheat but is also found in many other plants such as barley, triticale, and rye (Yang et al. 2015a). To control the disease and reduce yield loss, considerable effort has been made to elucidate the underlying mechanism of *G. graminis* var. *tritici* pathogenicity and its interaction with the host (Daval et al. 2011; Puga-Freitas et al. 2017; Yang et al. 2015b). The molecular mechanisms involved in plant response to pathogenic fungi are relatively well documented for fungi such as *Botrytis cinerea*, *Fusarium oxysporum*, *Parastagonospora nodorum*, and *Zymoseptoria tritici* (Berrocal-Lobo and Molina 2008; Duba et al. 2018; Williamson et al. 2007). In contrast, very little is known about the molecular response of wheat to *G. graminis* var. *tritici* infection. From previous transcriptomic studies, it was suggested that several genes involved in signal transduction, including pathogen-associated molecular patterns (PAMPs), are differentially expressed in *G. graminis* var. *tritici*-infected wheat as compared with controls (Guilleroux and Osbourn 2004; Puga-Freitas et al. 2017).

†Corresponding authors: C. Liu; chliu@nju.edu.cn and Y. Xue; xueyr@nju.edu.cn

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Currently, control of take-all mainly relies on cultural practices such as crop rotation with nonhost plants and utilization of ammonium fertilizers to make the wheat rhizosphere unfavorable to *G. graminis* var. *tritici* (Cook 2003). However, due to the absence of plant genetic resistance and efficient fungicides against the disease, it is increasingly believed that the application of biological control agents such as *Bacillus subtilis*, *Bacillus velezensis*, and *Pseudomonas fluorescens* may provide alternative management strategies to control the disease (Daval et al. 2011; Kang et al. 2018; Yang et al. 2015a). Our previous research demonstrated that *B. velezensis* CC09, an endophytic biocontrol bacterium, has unique disease prevention capability. It not only can lead to the production of broad-spectrum antifungal metabolites but also directly inhibits fungal growth in vitro and in vivo, and can be transmitted in plants, through stalks, from root to leaf. Like a “vaccine”, it can be applied once on the root to induce plant resistance to control both root take-all and leaf spot blotch (*Bipolaris sorokiniana*) diseases at the same time (Cai et al. 2017; Kang et al. 2018). However, the molecular mechanism of *B. velezensis*-mediated resistant against fungal disease in wheat is still unknown.

Despite genome complexity, several experiments have been conducted to understand possible response mechanisms of wheat challenged with beneficial bacteria (i.e., *Azospirillum brasilense* and *B. amyloliquefaciens*) or pathogenic fungi (i.e., *Botrytis cinerea*, *F. oxysporum*, and *G. graminis* var. *tritici*), and a number of genes or proteins involved in diverse plant defense responses mediated by salicylic acid (SA), jasmonic acid (JA), or ethylene (ET) have been identified using transcriptomic and proteomic techniques (Berrocal-Lobo and Molina 2008; Erayman et al. 2015; Puga-Freitas et al. 2017; Saia et al. 2015; Souza et al. 2014; Srivastava et al. 2016; Williamson et al. 2007; Xin et al. 2012; Zhang et al. 2018). However, to date, no study has been performed to understand the precise molecular mechanism of wheat in response to *B. velezensis*. Furthermore, current knowledge regarding the response of plants to either beneficial or pathogenic infections is only based on studying the interaction between plants and microbes using single host–single strain model systems rather than host–multiple microbe systems (Engelmann et al. 2011; Gonzalez et al. 2018). No studies have been carried out to understand the mechanism of wheat response to both beneficial and pathogenic microorganisms, a scenario more akin to what is found in natural agriculture ecosystems.

Plants employ diverse defense mechanisms at local or systemic levels to modify the environments in which they live. The first line of defense, defined as structural constitutive resistance, is mediated by physical barriers (i.e., cuticle, suberin deposition, and cell wall lignification) and the production of antagonistic chemicals (Jain et al. 2018; Piasecka et al. 2015). The second line of defense comprises systemic acquired resistance (SAR) and induced systemic resistance (ISR), mediated by the plant innate immune system against a variety of pathogens. SAR is a pathogen-induced defense system activated by many endogenous signals such as SA, pipecolic acid, N-hydroxy-pipecolic acid, and azelaic acid/glycerol 3-phosphate in plants upon pathogen priming (Chen et al. 2018; Hartmann et al. 2018; Klessig et al. 2018; Spoel and Dong 2012). In contrast to SAR, ISR is initiated by beneficial microbial priming (i.e., endophytes or plant-growth-promoting rhizobacteria [PGPR]), which triggers JA- and ET-mediated signaling pathways and enhances disease resistance against pathogen attack (Gond et al. 2015; Jain et al. 2018; Pieterse et al. 2014). As such, JA- or ET-mediated ISR is also the main mechanism for indirect control of disease by biocontrol agents (Bano and Muqarab 2017; Mhlango et al. 2018; Stringlis et al. 2018). Thus, ISR offers prospect as a plant protection system

against pathogens to be used in modern agriculture management systems to at least reduce the amount of chemical pesticides. However, it is not yet clear whether SAR or ISR plays a more important role in disease control when plants are cocolonized by biocontrol bacteria and pathogens. Furthermore, it remains to be determined whether the structural constitutive resistance of plants can be enhanced by colonization of biocontrol bacteria to reduce the damage caused by pathogens.

B. velezensis CC09 was an endophytic biocontrol bacterium isolated from the healthy *Cinnamomum camphora* leaf tissue (Cai et al. 2016). Our previous studies showed that *B. velezensis* CC09 has the ability to colonize almost every part of wheat root, including the epidermis, cortex, and xylem vessels, and even migrated to stems and leaves, resulting in 66.67% disease-control efficacy (DCE) of take-all and 21.64% DCE of spot blotch (Kang et al. 2018). The aim of this follow-up study was to elucidate the molecular mechanism in wheat roots of the response to a *B. velezensis*, a take-all-causing pathogen *G. graminis* var. *tritici*, and their combined colonization using RNA-seq, iTRAQ (isobaric tags for relative and absolute quantification), and biochemical analysis techniques. The possible synergistic interaction between *B. velezensis* and *G. graminis* var. *tritici* inside root tissues and its potential effect on the defense response system of wheat were explored in detail.

RESULTS

Transcriptional profiles of wheat in response to *B. velezensis*, *G. graminis* var. *tritici*, and *B. velezensis* + *G. graminis* var. *tritici* colonization.

Screening of differentially expressed genes. The RNA-seq data were well validated by quantitative real-time PCR (qRT-PCR) analysis, indicating that the data could be used for the follow-up analysis (Supplementary Table S1; Supplementary Fig. S1). A comparative transcriptomic analysis was undertaken to explore the molecular mechanism of wheat roots in response to the biocontrol bacterium *B. velezensis*, the take-all pathogen *G. graminis* var. *tritici*, and their combination (*B. velezensis* + *G. graminis* var. *tritici*). In general, the expression of most genes remained unchanged among the total of 80,566 detected genes (reads per kilobase per million [RPKM] > 0). In total, 10,780 genes (4,831 upregulated and 5,945 downregulated), 13.4% of the total expressed genes, were identified as differentially expressed genes (DEGs) caused by the combination of *B. velezensis* and *G. graminis* var. *tritici* (Supplementary Table S2). In contrast, only 3,127 (1,355 upregulated and 1,772 downregulated) (3.9% of the total expressed genes) and 5,678 (2,833 upregulated and 2,845 downregulated) (7.0% of the total expressed genes) DEGs were identified in the *B. velezensis*- and *G. graminis* var. *tritici*-colonized wheat roots, respectively (Supplementary Fig. S2). The number of DEGs caused by *G. graminis* var. *tritici* infection was 1.8 times that of the *B. velezensis*-colonized sample. However, the number of DEGs in combination-colonized samples was 3.4 and 1.9 times those of the *B. velezensis*- and *G. graminis* var. *tritici*-colonized samples, respectively. Moreover, both the up- and downregulated DEGs of wheat roots that were cocolonized by *B. velezensis* and *G. graminis* var. *tritici* were much higher than those of the *B. velezensis*- or *G. graminis* var. *tritici*-colonized wheat roots. For instance, the number of upregulated DEGs of the cocolonized sample was 3.6 and 1.7 times those of the *B. velezensis*- and *G. graminis* var. *tritici*-colonized samples, respectively; the number of downregulated DEGs of the cocolonized sample was 3.4 and 2.1 times higher than those of the *B. velezensis*- and *G. graminis* var. *tritici*-colonized samples, respectively. Meanwhile, the number of up- and downregulated

DEGs of *G. graminis* var. *tritici*-colonized wheat roots was 2.1 and 1.6 times those of *B. velezensis*-colonized wheat roots, respectively. In addition, a statistical analysis revealed that, when wheat was subjected to *G. graminis* var. *tritici* or the combined infection, the expression level of most genes were induced with a higher number of extreme transcriptional changes (>10-fold) compared with *B. velezensis* treatment. In conclusion, changes in gene expression levels were more drastic when wheat was challenged by two microorganisms than when treated with a single microorganism, especially for the beneficial microorganism.

Clustering of DEGs. Hierarchical cluster analysis was conducted using 13,400 DEGs that showed significant differential expression in at least one sample (Fig. 1). The analysis clearly revealed that the endophytic *B. velezensis*-colonized root sample and the control samples clustered into one branch and the pathogenic *G. graminis* var. *tritici*- and combination-colonized samples clustered into another branch. Cluster analysis of the RPKM data showed that the gene expression pattern could be divided into seven clusters based on the expression levels of the DEGs in *B. velezensis*-, *G. graminis* var. *tritici*-, and combination-colonized roots versus the microbe-free control (Supplementary Table S3); cluster D was enriched in transcripts that showed large fold changes in expression between *B. velezensis*-colonized and control roots, while cluster G represented the fold changes between *G. graminis* var. *tritici*-colonized and control roots. Clusters E and F contained DEGs with very high expression levels in combination-colonized roots compared with the control.

Discrete transcriptome responses to bacterial or fungal colonization. The short time-series expression miner (STEM) platform was used to analyze the transcriptome dynamics in response to *B. velezensis*, *G. graminis* var. *tritici*, and combination colonization on total DEGs. In total, four important temporal gene expression profiles were obtained (Fig. 2A). Most of the upregulated genes (2,278) were clustered into profile 17, and many DEGs in this profile are involved in

the biosynthesis of secondary metabolites (i.e., terpenoid backbone, diterpenoid, stilbenoid/diarylheptanoid/gingerol, and flavonoid biosynthesis) as well as other metabolic pathways associated with plant defense such as the plant–pathogen interaction pathway (Supplementary Figs. S3 and S4). Genes encoding pathogenesis-related (PR) proteins and pattern recognition receptors (PRRs) involved in PAMP-triggered immunity (PTI), calcium influx genes, hormone regulation genes, respiratory burst oxidase genes, and mitogen-activated protein kinase cascade genes were all enhanced in response to *B. velezensis*, *G. graminis* var. *tritici*, and both combined. Based on the predicted nucleotide-binding site leucine-rich repeat (NBS-LRR) family genes in the wheat genome (Peng and Yang 2017), we conducted further analysis of the DEGs encoding NBS-LRR proteins in *B. velezensis*-, *G. graminis* var. *tritici*-, and combination-colonized wheat roots. The distribution of up- and downregulated NBS-LRR family DEGs was calculated and presented in a Venn diagram (Fig. 2B). Expression of 13 such DEGs was elevated post *B. velezensis* inoculation, after which it returned to normal, while the levels of 46 additional DEGs increased following treatment with combination. In conclusion, the combined interaction of *B. velezensis* and *G. graminis* var. *tritici* elicited more significant changes in DEGs involved in both PTI and effector-triggered immunity (ETI) defense programs than that of each single microorganism.

Profile 4 contained the most transcripts (3,368); most of genes in this profile as well as profiles 1 and 10 were largely involved in primary metabolism such as the metabolism of carbohydrates (i.e., glyoxylate, dicarboxylate, galactose, starch, and sucrose), lipids, amino acids, and photosynthesis, and these genes were dynamically downregulated. The upregulation of genes involved in wheat defense pathways and the downregulation of genes involved in primary metabolism indicate that readjustment of plant primary metabolism likely contributes to plant defense responses against pathogen attack.

Fig. 1. Hierarchical clustering analyses of differentially expressed genes. According to the three sample's reads per kilobase per million, red indicates high expression of the gene, green indicates low expression of genes. Bv, Ggt, and Bv+Ggt = *Bacillus velezensis*, *Gaeumannomyces graminis* var. *tritici*, and their combination, respectively.

Expression of genes encoding JA and SA synthases. JA is a major phytohormone induced by plants to elicit systemic resistance against pathogens. Lipoxygenases (LOX), allene oxide synthase, allene oxide cyclase, and oxo-phytodienoate reductase (OPR) are the main enzymes involved in the biosynthesis of JA (Supplementary Fig. S5). In this study, eight DEGs involved in LOX biosynthesis were identified, and 87.5% of these genes were upregulated in *B. velezensis*-colonized sample; four LOX genes were found in the *G. graminis* var. *tritici*-colonized sample and 75.0% of these genes were upregulated; and six LOX genes were identified in the combination-colonized sample but only 33.3% of them were upregulated (Table 1). In other words, most of the LOX genes were downregulated in the combination-colonized sample. A similar regulatory pattern of DEGs was found for the OPR genes.

Phenylalanine ammonia lyase (PAL) is a key enzyme involved in SA biosynthesis. In total, 13 DEGs encoding PAL enzymes were identified and all of these genes were upregulated in the pathogenic *G. graminis* var. *tritici*-colonized roots; 2 were found in the combination-colonized sample, with all upregulated; and 3 were identified in the *B. velezensis*-colonized sample, with all downregulated (Table 1). These data suggest that the biocontrol bacterium *B. velezensis* might have the ability to stimulate JA production in wheat plants, whereas the pathogen *G. graminis* var. *tritici* might induce the accumulation of JA and SA. These results also suggest that JA content might be reduced whereas SA content might be increased in wheat cocolonized by *B. velezensis* and *G. graminis* var. *tritici*.

Expression of JA- and SA-responsive genes in wheat roots. The plant hormones JA and SA are important regulators of induced defense mechanisms. We next sought to determine how the defense pathways mediated by JA and SA respond in *B. velezensis*-, *G. graminis* var. *tritici*-, or combination-colonized samples. *PR* protein 1 (*PR1*) and *PDF1.2*, marker resistance genes representing SA and JA pathways, respectively, were investigated (Vos et al. 2015). The transcriptome data

demonstrated that, regardless of whether the wheat roots were colonized by a single species or two combined, or a beneficial or pathogenic microorganism, *PR1* genes were always upregulated (Table 2). In all, 12 and 13 *PR1* genes were significantly changed for *G. graminis* var. *tritici* and combination samples, respectively. In contrast, only six *PR1* DEGs were identified in *B. velezensis*-colonized samples. Additionally, in the *G. graminis* var. *tritici* and combination samples, there were four and five *PR1* genes with an approximately four- to sevenfold change,

Table 1. Profile of gene expression (up/down) involved in the biosynthesis of jasmonic acid (JA) and salicylic acid (SA) in wheat roots

Plant hormone	Gene name ^a	<i>Gaeumannomyces</i>		
		<i>Bacillus velezensis</i>	<i>graminis</i> var. <i>tritici</i>	Both organisms combined
JA	LOX	7/1	3/1	2/4
JA	AOS	1/0	–	0/2
JA	AOC	–	–	1/2
JA	OPR	5/3	7/2	5/7
SA	PAL	0/3	13/0	2/0

^a LOX = lipoxygenases, AOS = allene oxide synthase, AOC = allene oxide cyclase, OPR = oxo-phytodienoate reductase, and PAL = phenylalanine ammonia lyase; – indicates that expression of the genes was not detected.

Table 2. Expression of marker resistance genes (up/down) that can represent jasmonic acid (JA)- and SA-mediated defense pathways in *Bacillus velezensis*-, *Gaeumannomyces graminis* var. *tritici*-, and combination-colonized roots

Gene name ^a	<i>B. velezensis</i>	<i>G. graminis</i> var. <i>tritici</i>	Combination
<i>PR1</i>	6/0	12/0	13/0
<i>PDF1.2</i>	0/1	0/5	0/6

^a *PDF1.2* = defensin-like protein gene and *PR1* = pathogenesis-related protein 1 gene.

Fig. 2. A, Expression patterns and **B,** numbers of differentially expressed genes encoding nucleotide-binding site leucine-rich repeat (NBS-LRR) proteins. Expression patterns of genes identified by transcriptome comparisons of treatments with *Bacillus velezensis* (Bv), *Gaeumannomyces graminis* var. *tritici* (Ggt), both combined (Bv+Ggt), and the control (CK). The Venn diagram illustrates the numbers of NBS-LRR family genes with upregulated (upward-pointing arrow) or downregulated (downward-pointing arrow) expression in *B. velezensis*-, *Gaeumannomyces graminis* var. *tritici*-, and combination-colonized wheat roots.

respectively, whereas, in the *B. velezensis* sample, there was a single *PR1* gene with an approximately one- to twofold change (Supplementary Table S4). When taking into account changes in SA content (Fig. 3), these results indicate that the SA-mediated resistance pathway is induced regardless of whether or not wheat roots are colonized by beneficial bacteria, pathogenic fungi, or both but the presence of the pathogen (*G. graminis* var. *tritici* sample) enhances this pathway and the combined interaction (combination sample) further induces it.

In contrast to the expression of *PR1* genes, the expression of the JA-signaling pathway marker *PDF1.2* gene was inhibited in all treatment groups (Table 2). Of the *PDF1.2* genes, only one (Traes_4DL_909858768) showed a significant (1.08-fold) change caused by the beneficial *B. velezensis*. In contrast, 60% (3 of 5) of *PDF1.2* genes showed an approximately three- to fourfold change in the *G. graminis* var. *tritici* sample, while 83% (5 of 6) showed an approximately three- to fourfold change in the combination sample. In summary, compared with *G. graminis* var. *tritici* infection, the expression of JA-responsive genes was attenuated in the combination sample.

Expression of genes involved in lignin biosynthesis. Deposition of lignin is thought to contribute to plant resistance by preventing the diffusion of pathogens (Bellincampi et al. 2014). To understand how lignification changes in response to single or combined, beneficial or pathogenic microorganisms in wheat roots, we next explored the expression changes of key genes involved in lignin biosynthesis. Based on previous studies, PAL, cinnamate 4-hydroxylase (C4H), and 4-coumarate CoA ligase (4CL) catalyze the first three steps in the phenylpropanoid pathway (Fig. 4A), producing precursors to lignin. Whereas genes encoding these enzymes were downregulated in the *B. velezensis* sample, all were found to be induced upon *G. graminis* var. *tritici* treatment (Fig. 4B). In the combination sample, although most of the genes encoding 4CL protein were downregulated, critical lignin biosynthesis genes encoding PAL and C4H were upregulated. This result indicated that precolonization of *B. velezensis* might lead to an increase of cell wall lignification, or it is possible that the interaction between *B. velezensis* and *G. graminis* var. *tritici* triggered the complex regulation of gene expression in planta.

Proteome profiles of wheat in response to *B. velezensis*, *G. graminis* var. *tritici*, and combination colonization.

To identify the differentially expressed proteins (DEPs) in wheat roots colonized by *B. velezensis*, *G. graminis* var. *tritici*,

and both combined relative to control plants, we performed a quantitative proteomic analysis based on iTRAQ technology. According to the standards of DEP identification (≥ 1.5 -fold cutoff, P value < 0.05), only eight proteins, no matter whether in *G. graminis* var. *tritici*- or in combination-colonized wheat roots, showed a significant difference from 783 detected proteins (Supplementary Table S5). However, no DEPs were identified in the *B. velezensis*-colonized wheat roots. Among these DEPs, five were regulated in both *G. graminis* var. *tritici*- and combination-colonized roots, including phosphoglycerate kinase, phosphoenolpyruvate carboxylase 2, aspartate aminotransferase, cyclophilin A, and a predicted protein (Supplementary Table S6). Three DEPs were specifically regulated in

Fig. 4. Effects of *Bacillus velezensis* (Bv), *Gaeumannomyces graminis* var. *tritici* (Ggt), and both combined (Bv+Ggt) on the biosynthesis of lignin. **A**, Overview of lignin biosynthesis. **B**, Heat map of lignin biosynthesis-related gene expression. PAL = phenylalanine ammonia lyase, C4H = cinnamate 4-hydroxylase, and 4CL = 4-coumarate CoA ligase.

Fig. 3. Effects of *Bacillus velezensis* (Bv), *Gaeumannomyces graminis* var. *tritici* (Ggt), and both combined (Bv+Ggt) on the biosynthesis of jasmonic acid (JA) and salicylic acid (SA). **A**, JA and **B**, SA concentration in wheat roots. Different letters indicate a significant difference between treated and control (CK) samples based on Fisher's least significant difference test at P value = 0.05.

G. graminis var. *tritici*-colonized wheat roots, including putative 26S proteasome non-ATPase regulatory subunit 3, 40S ribosomal protein S16, and Serpin-ZX. Likewise, three DEPs were specifically regulated in combination-colonized roots, including endoplasmic-like protein, 40S ribosomal protein S6, and serine hydroxymethyltransferase, which might contribute to wheat resistance improvement.

Comparison analysis of transcriptome and proteome data.

In total, 129 proteins were identified for which corresponding transcripts were represented in the RNA-seq data. The comparison analyses of transcript and protein data from *B. velezensis*, *G. graminis* var. *tritici*, and combination treatments revealed that the Pearson correlation (r) was 0.0196, 0.0927, and 0.0116, respectively (Fig. 5).

Contents of phytohormones and lignin in wheat roots.

To validate the regulatory effect of *B. velezensis*, *G. graminis* var. *tritici*, and combination colonization on the biosynthesis of phytohormones, we measured the concentrations of JA and SA in wheat roots (Fig. 3). Compared with that of controls (40.69 ng g^{-1}), the amount of JA was significantly increased in *B. velezensis*-colonized (75.97 ng g^{-1}) and *G. graminis* var. *tritici*-colonized (49.37 ng g^{-1}) wheat roots but decreased in the combination-colonized roots (32.95 ng g^{-1}). In contrast, the concentration of SA was not significantly altered in *B. velezensis*-colonized roots ($1,148.52 \text{ ng g}^{-1}$) but was significantly increased in *G. graminis* var. *tritici*-colonized ($1,499.90 \text{ ng g}^{-1}$) and combination-colonized ($2,030.24 \text{ ng g}^{-1}$) samples relative to that of the control ($1,197.32 \text{ ng g}^{-1}$). These results are consistent with the data obtained from the transcriptomic studies (Table 1).

Lignin is an aromatic polymer that is mainly deposited in secondary thickened cell walls, where it provides strength and imperviousness against pathogen infection. To understand the role of lignin in response to colonization of single or combined, beneficial or pathogenic microorganisms, lignin content was investigated. A comparison of wheat root cross-sections demonstrated that inoculation of *B. velezensis*, *G. graminis* var. *tritici*, or both combined resulted in more lignified tissue compared with noninoculated plants (Fig. 6). However, lignin content was increased in the presence of the pathogen (Fig. 6C and D), with the highest levels observed in the combination sample, suggesting that the coinfection by *B. velezensis* and *G. graminis* var. *tritici* further strengthens the secondary wall thickness.

DISCUSSION

This is the first comprehensive report of wheat, a hexaploid plant, aiming to analyze changes in transcription and translation in response to single or combined, beneficial or pathogenic microorganisms. Based on the current study, compared with the *G. graminis* var. *tritici* and combination samples, there were not only fewer DEGs or DEPs but also relatively mild gene expression levels on the whole in the *B. velezensis* sample, especially for the plant-pathogen interaction pathway genes, NBS-LRR family genes, and PR1 proteins. Downregulation of root immune responses has also been described for ISR-inducing PGPR such as *B. subtilis* FB17 and *P. fluorescens* WCS417r (Millet et al. 2010; Verhagen et al. 2004). Lakshmanan et al. (2013) suggested that beneficial microbes may partially block immune responses in *Arabidopsis thaliana* roots and support efficient root colonization and establishment of rhizosphere microbial communities. To achieve similar effects and establish a mutualistic interaction with the plant, beneficial *B. velezensis* may therefore need to prevent, weaken, or

overcome host immune responses in wheat roots. Our results showed that combined inoculation of wheat roots elicited more large-scale transcriptional changes, especially for genes associated with plant-interaction pathways that are closely related

Fig. 5. Correlation graph of transcripts and associated proteins. Bv, Ggt, and Bv+Ggt = *Bacillus velezensis*, *Gaeumannomyces graminis* var. *tritici*, and their combination, respectively.

to plant defense. According to the hypothesis of trade-off between constitutive and inducible resistance traits in plants (Morris et al. 2006), a wheat plant having more potential to induce DEGs may have improved resistance. Based on the current data, we also discovered that *B. velezensis* triggered a PTI-mediated defense system in wheat roots, which is consistent with previous studies (Lopez-Gomez et al. 2012; Trdá et al. 2014). To our knowledge, however, the mechanisms that allow beneficial bacteria to colonize plants are still limited. A few studies on interactions between *Burkholderia phytofirmans* and *A. thaliana* (Trdá et al. 2014) and *Sinorhizobium meliloti* and *Lotus japonicus* (Lopez-Gomez et al. 2012) have demonstrated that these PGPR strains triggered PTI in a manner dependent on flagellin-sensitive 2 (FLS2). However, there are many differences in the interactions between various bacterial species and plants. For example, cooperation between *Azospirillum* spp. and rice cultivars depends on the genetic nature of both the bacterium and the plant (Drogué et al. 2014). Unlike previous studies, the present work revealed that the beneficial *B. velezensis* triggered an ETI defense program by activating a large

number of resistance (*R*) genes assigned to the NBS-LRR family. Moreover, we noticed that, after *G. graminis* var. *tritici* infection, roots from plants pretreated with *B. velezensis* elicited more drastic changes in important PRR genes triggered by PAMPs, such as brassinosteroid insensitive 1-associated kinase 1 (BAK1), FLS2, and chitin elicitor receptor kinase (CERK), which can transduce signals that trigger PTI (Dodds and Rathjen 2010). Also, *B. velezensis*-treated plants were able to rapidly activate the expression of *R* genes involved in ETI when the plants were challenged by *G. graminis* var. *tritici*. These results suggest that precolonization by *Bacillus* spp. not only sensitizes the PTI but also the ETI for enhanced defense in wheat roots. Perhaps for this reason, the primed state induced by a PGPR strain is often invisible in unchallenged plants (Pieterse et al. 2014). Therefore, when the ISR-expressing wheat plants were challenged with the pathogen *G. graminis* var. *tritici*, larger transcriptional changes may have occurred than in nonprimed plants.

When compared with inoculation by a single microorganism, the results we obtained for combined inoculation (bacterium

Fig. 6. Effects of lignin structure on wheat colonized by *Bacillus velezensis*, *Gaeumannomyces graminis* var. *tritici*, and their combination. Transverse sections of adventitious roots under different treatments. **A**, Control; **B**, *B. velezensis*; **C**, *G. graminis* var. *tritici*; and **D**, both organisms combined. The red stain indicates the location of lignin structure with phloroglucinol-HCl staining.

plus fungus) are more likely to reflect the actual transcriptional and translational changes that might be expected under natural agriculture conditions. Furthermore, our work sheds light how wheat responds to beneficial and pathogenic microorganisms simultaneously. We found that the changes in gene expression elicited by combination treatment was more similar to that of the individual infection by *G. graminis* var. *tritici* (Figs. 1 and 6B). According to a recent study described by Kang et al. (2018), although inoculation of *B. velezensis* can alleviate wheat disease, it does not completely prevent infection by the pathogen *G. graminis* var. *tritici*. Therefore, like the pathogen infection individually, the combination of *B. velezensis* and *G. graminis* var. *tritici* ultimately gave rise to take-all disease (Kang et al. 2018). Such an outcome contributed to the similar expression pattern between *G. graminis* var. *tritici* and combination samples but it also indicated that, when wheat roots are challenged by both beneficial and pathogenic microorganisms, the plants most likely prioritize response mechanisms to resist the invasion of the pathogen.

Plants can activate defense responses to both pathogenic and beneficial microorganisms. These defense responses are largely modulated through hormone production. The plant hormones ET, JA, and SA are important regulators of induced defense mechanisms (Vos et al. 2015). Previous studies have found that treatment with ISR-inducing PGPR did not lead to upregulation of JA- or ET-responsive genes in plants; however, when challenged with pathogens, these plants exhibited induced expression of the JA-responsive gene *PDF1.2* (Pieterse et al. 2014; Van Oosten et al. 2008), which suggests that pretreatment with the beneficial strain can prime plant immune system sensitivity. However, in this study, unlike other PGPR strains, colonization of *B. velezensis* promoted the accumulation of JA in wheat roots. Moreover, the marker gene *PDF1.2* was not induced but was, instead, inhibited. In addition, when wheat was subsequently challenged with the pathogen *G. graminis* var. *tritici*, the degree of inhibition of *PDF1.2* gene expression was aggravated compared with that during *G. graminis* var. *tritici* infection individually. These data appear to indicate that the colonization of *B. velezensis* caused the JA-induced resistance pathway to be inactivated. However, in the current study, six *PR1* genes were all induced by *B. velezensis* treatment. *PR1* is a marker gene for *Arabidopsis* SAR (Dong 2004), indicating that the SA-mediated SAR defense response was also triggered by *B. velezensis*. In addition, *B. velezensis*-pretreated plants rapidly accumulated SA and the expression of *PR1* was slightly higher than for *G. graminis* var. *tritici* infection individually when the wheat plants were challenged with *G. graminis* var. *tritici*. Based on the current data, we suggest that, similar to ISR triggered by JA or ET, *B. velezensis*-mediated SAR in wheat also requires sensitivity to SA but is not accompanied by an increase in its production (Pieterse et al. 2000; van Wees et al. 1999).

Compared with the control, *G. graminis* var. *tritici* infection led to a larger change in SA than JA accumulation. In addition, whereas the SA-responsive gene *PR1* was induced, the JA-responsive gene *PDF1.2* was inhibited. Based on our evaluation of hormone content and the expression of resistance marker expression, we conclude that *G. graminis* var. *tritici* infection induces a stronger SA-mediated resistance response than that of JA, suggesting a more important role for SA than JA in the plant defense against *G. graminis* var. *tritici* infection. However, there are differences between our results and previous findings. SA-dependent defenses are generally effective against biotrophic pathogens, whereas JA-dependent defenses are generally effective against necrotrophic pathogens and herbivorous insects (Glazebrook 2005). According to our understanding, this principle is not applicable in all circumstances because many factors (i.e., ET, JA, and SA) contribute to sensitizing a

plant's immune system. Furthermore, plants employ different strategies to resist infection by various pathogens, often utilizing the most efficient approach (Shigenaga et al. 2017; Vos et al. 2015). Selecting an effective defense response is generally determined by the genetic characteristics of the plants and the pathogens themselves (Robert-Seilanianantz et al. 2011).

Studies on the antagonistic relationship between SA- and JA-signaling pathways have focused on dicotyledonous plants, particularly the model species *A. thaliana* (Shigenaga et al. 2017; Vos et al. 2015). Overall, our results reveal that the antagonistic relationship between SA and JA in wheat is not affected by single or combined, beneficial or pathogenic microorganisms (Fig. 3; Table 2) but, instead, is enhanced or attenuated in response to different microorganisms. When wheat was coinfectd with the beneficial *B. velezensis* and the pathogen *G. graminis* var. *tritici*, it further enhanced the SA-mediated signaling pathway and weakened the JA-mediated pathway. Furthermore, coinoculation of *B. velezensis* and *G. graminis* var. *tritici* resulted in changes in gene expression more similar to that of *G. graminis* var. *tritici* individually (Fig. 1). Therefore, in order to more effectively resist the pathogen *G. graminis* var. *tritici*, a more efficient approach might be chosen which, as this study suggests, is likely to involve the SA-mediated pathway. However, due to the antagonistic relationship between JA and SA, enhancement of the SA pathway will likely attenuate the JA pathway, which is viewed as a cost-saving strategy that has evolved in plants to reduce allocation costs by repression of unnecessary defenses, thereby minimizing trade-offs between plant defense and growth (Vos et al. 2015).

Deposition of lignin has been shown to enhance plant resistance to infection by preventing the diffusion of invading pathogens (Bellincampi et al. 2014). Lignin is responsible for preventing cell-wall degradation by pathogen-produced enzymes and for limiting the diffusion of pathogen-produced toxins (Sattler and Funnell-Harris 2013). Generally, a positive correlation was found between lignin accumulation and the severity of disease caused by pathogen infection (Ma et al. 2018). Due to the biocontrol and induced wheat resistance properties of *B. velezensis*, the disease severity in wheat roots inoculated with both *B. velezensis* and *G. graminis* var. *tritici* became weaker than that of *G. graminis* var. *tritici* infection alone (Kang et al. 2018). However, the presence of *B. velezensis* further enhanced lignification that resulted from *G. graminis* var. *tritici* inoculation, which is thought to effectively protect adjacent plant cells from infection (Sattler and Funnell-Harris 2013). We suggest that the enhancement of lignin accumulation is caused by the additive effect of the beneficial bacteria *B. velezensis* and the pathogen *G. graminis* var. *tritici*. In addition, phenylpropanoid metabolism involves a complex series of branching biochemical pathways that provide plants with diverse compounds, many of which are used as structural cell components (lignin, suberin, and other cell-wall-associated phenolics), pigments (flavonoids and anthocyanins), immunity signals (SA), and toxins (coumarins and furanocoumarins) (Khanam et al. 2018; Vogt 2010). PAL is a key enzyme that catalyzes an early reaction in the production of phenylpropanoids. Therefore, the upregulation of PAL that we observed in the combination coinoculation experiment likely reflects the importance of this enzyme in the biosynthesis of not only SA but also lignin.

According to the strict criteria of ≥ 1.5 -fold cutoff and *P* value < 0.05, only a few DEPs were identified in *G. graminis* var. *tritici*- or combination-colonized wheat roots, which might be due to the sensitivity of proteome analysis, availability of protein database, and posttranslational modifications of proteins (Pascovici et al. 2013). Through literature investigation, we found that there is no unified standard for the identification of DEPs. For example, Chen et al. (2016) used fold change and *P* value to

identify the DEPs in cotton roots and leaves under salt stress, while Wang et al. (2014) and Sun et al. (2016) used fold change and error factor to distinguish DEPs in rice roots under aluminum stress and different cultivars of ginseng, respectively. Zargar et al. (2015) and Wang et al. (2016) even used fold change only to identify the DEPs in *A. thaliana* and *Triticum polonicum* roots in response to metal deficiency based on iTRAQ analysis. In addition, although fold change is applied to proteome studies, the level of fold change also varies in different studies, such as 1.1 (Wang et al. 2014), 1.2 (Chen et al. 2016), 1.3 (Gao et al. 2016), 1.4 (Sun et al. 2016), 1.5 (Huang et al. 2013; Wang et al. 2016), and 2 (Zargar et al. 2015). Therefore, how to define significantly differentially expressed proteins is worth further exploration.

Consistent with many other studies, the correlations between transcriptome and proteome data are very low (0.0116 to 0.0927) in *B. velezensis*-, *G. graminis* var. *tritici*-, and combination-colonized wheat roots. It has been explained that the low correlations between transcriptome and proteome data are related to the complexity of the process from gene transcription to protein synthesis in wheat roots (Fournier et al. 2010; Gedeon and Bokes 2012; Huang et al. 2013). For example, Huang et al. (2013) used RNA-seq and iTRAQ to study the nitrogen starvation response of *Synechocystis* sp. PCC 6803, and found that the correlation coefficients (r) of transcriptome and proteome data at 24 and 48 h were 0.04 and -0.001, respectively. They attributed this result to possible posttranscriptional, translation, or posttranslational regulation of mRNA. In addition, the iTRAQ technology mainly relies on the ability to match peptide tandem mass spectra to sequences from the protein databases (Pascovici et al. 2013). Due to the large genome size (17 gb) and high proportion of repetitive sequences (>80%), hexaploid wheat contains a complex and huge protein database. When comparing protein homology, it is easy to have ambiguous matching, which affects protein recognition and quantitative analysis. Thus, both biological reasons (e.g., pretranslational or post-translational regulation of gene expression) and technical reasons (i.e., spectral mismatches in wheat's complex genome) may lead to only a small number of proteins identified by iTRAQ and a low correlation between proteomic results and transcriptional results.

Conclusion.

In this study, we make the first report of hexaploid wheat response to *B. velezensis* CC09, *G. graminis* var. *tritici*, and both microorganisms combined through the comparative analysis of transcriptome and proteome. We showed that the SA-dependent type of ISR is also triggered by beneficial *B. velezensis*, and the enhancement of SA-dependent defense response (i.e., lignin deposition) by the preinoculated *B. velezensis* is shown to reduce the level of induced JA-dependent defense against subsequent pathogen *G. graminis* var. *tritici* infection. In addition, wheat roots pretreated by *B. velezensis* not only exhibit larger transcriptional or translational programming than that of a single microorganism but also possess a more sensitive plant innate immunity system when the wheat roots were challenged with subsequent pathogen *G. graminis* var. *tritici*. In short, the current study provide a deeper understanding of the relationships in the hexaploid wheat-beneficial strain-pathogen fungus, which may lead to new biocontrol methods and help alleviate wheat crop-related disease issues.

MATERIALS AND METHODS

Wheat, bacterium, and fungus.

The winter wheat cultivar Sumai 188, purchased from Jiangsu Academy of Agricultural Sciences, Nanjing, China,

was used in this study. The endophytic biocontrol bacterium *B. velezensis* CC09 (strain deposit number CICC 24093; GenBank accession number CP015443) and take-all-causing pathogen *G. graminis* var. *tritici* were used in this study. *B. velezensis* was cultured in liquid Luria-Bertani medium at 37°C in a rotatory shaker (200 rpm). After growth for 12 h, sample inocula (1×10^8 CFU ml⁻¹) were centrifuged at $7,000 \times g$ for 5 min, and resuspended in sterile distilled water. *G. graminis* var. *tritici* was cultured at 25°C on potato dextrose agar medium for 10 days.

Evaluation of global responses to single and combined microorganisms in wheat roots.

Wheat seeds were surface disinfected and germinated as described by Kang et al. (2018). The germinated wheat seeds were cultured on a sterilized raising plate containing 120 ml of half-strength Murashige and Skoog medium (catalog number M0222.0050; Duchefa Biochemie) and incubated at 25°C under 14 h of light and 10 h of darkness for 7 days. Roots of wheat seedlings were inoculated with 30 ml of *B. velezensis* inoculum or the same amount of sterile distilled water. Five days post-inoculation with *B. velezensis*, the seedlings were placed on a water-agar plate and each root of the seedlings was inoculated with a plug of mycelia that was cut from the colony margin of *G. graminis* var. *tritici*. Nonpathogenized, nonbacterized, and watered control plants were also included. This experiment can be simply described as four treatments: (i) control (CK), roots inoculated with sterile water; (ii) *B. velezensis*, roots inoculated with beneficial bacterium *B. velezensis* only; (iii) *G. graminis* var. *tritici*, roots inoculated with take-all pathogen *G. graminis* var. *tritici* only; and (iv) combination, roots inoculated with both *B. velezensis* and *G. graminis* var. *tritici*. Seedlings were grown under a controlled environment (light intensity of $200 \mu\text{mol m}^{-2} \text{s}^{-1}$, cycle of 14 h/day and 10 h/night, at a constant temperature of 25°C and a relative humidity of 50%). At 8 days after bacterial inoculation, approximately 30 seedlings were collected and stored at -80°C prior to RNA or protein extraction. Fresh samples were used for biochemical assays, including the observation of lignin structure and determination of plant hormones. Each experiment was repeated three times, with 10 replicates per treatment.

Microscopic observation of lignification of root tissue.

Lignification is an indicator of plant defense against pathogens. To understand whether the combined inoculation of *B. velezensis* and *G. graminis* var. *tritici* can enhance the lignification of root tissue, we conducted histological analysis based on a previously described staining method with small modification (Zhu et al. 2018). Briefly, the cross section of the root samples was directly prepared by the bare-handed method. Then, a few drops of acidified phloroglucinol ethanol solution (2% phloroglucinol dissolved in ethanol, mixed with 1% HCl) were poured on the sections of the roots, and immediately observed with a light microscope (CX40; Shunyu).

Quantification of phytohormone.

A 1.0-g root sample frozen in liquid nitrogen was used for extraction and quantification of phytohormones JA and SA using the method described by Cheng et al. (2017). An enzyme-linked immunosorbent assay kit was used to quantify the levels of the phytohormones JA and SA, purchased from Jiangsu Meibiao Biological Technology Company Limited. All measurements were replicated three times.

Total RNA extraction and RNA-seq analysis.

Roots of five plants were pooled for total RNA extraction using an RNAPrep Pure Plant Kit (catalog number DP441;

Tiagen) following the manufacturer's instructions. The purity and integrity of the obtained RNA were detected using an Agilent 2100 Bioanalyzer RNA chip (Agilent Technologies). In total, four RNA-seq libraries were constructed, representing the four treatments of CK, *B. velezensis*, *G. graminis* var. *tritici*, and combination, and sequenced on an Illumina HiSeq™ 4000 platform following a default Illumina stranded RNA protocol (Personalbio). Each library produced approximately 1,800 million 150-bp reads yielding more than 25 gb of sequenced data so as to detect the low-abundance mRNA. Filtered clean reads were mapped to reference genome of *T. aestivum* (The International Wheat Sequencing Consortium [IWGSC] 2014) using SOAPaligner/SOAP2 software (Li et al. 2009), and deposited in the NCBI Sequence Read Archive under BioProject ID PRJNA485739. Transcript expression was normalized to RPKM for quantification of each gene. Genes exhibited significant changes in expression as determined through the DESeq R package (Love et al. 2014). The resulting *P* values were adjusted using the Benjamini and Hochberg approach for controlling the false discovery rate. Genes with an adjusted *P* value < 0.05 found by DESeq and absolute value of log₂ratio ≥ 1 were assigned as DEGs. The DEGs were annotated using the Kyoto Encyclopedia of Genes and Genomes (KEGG) database. STEM software was used to identify the significantly enriched expression patterns (*P* value < 0.05) in *B. velezensis*-, *G. graminis* var. *tritici*-, and combination-colonized wheat roots. The RPKM value of CK, *B. velezensis*, *G. graminis* var. *tritici*, and the combination was used as the input dataset, and we performed the trend analysis to identify the significantly enriched expression patterns (*P* value < 0.05) on the STEM platform. The parameters were set on the STEM platform, including the input of log₂-transformed RPKM values, a maximum number of model profiles of 20, and a maximum unit change of 2.

Total protein extraction and iTRAQ analysis.

A 0.5-g sample of root tissue from each treatment was ground in liquid nitrogen and extracted using 4 ml of extraction buffer (50 mM Tris-HCl [pH 7.4], 100 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, 1 mM EDTA, 1% Triton X-100, 1% sodium deoxycholate, and 2% sodium dodecyl sulfate) following the method described by Wang et al. (2014), with minor modification. The mixture was purified using acetone, and the purified proteins were dissolved in a 7M urea solution and quantified using a QuantiPro BCA Assay Kit (TaKaRa Bio Inc.) following the manufacturer's instructions. The obtained proteins were stored at -80°C for iTRAQ analysis, including iTRAQ labeling, SCX chromatography fractionation, and liquid chromatography-electrospray ionization tandem mass spectrometry (MS/MS) analysis with a 5600 TripleTOF platform (AB SCIEX) following the method described by Yang et al. (2013). The MS/MS data were analyzed through a search and quantitative analysis against the nonredundant protein database of the hexaploid wheat genome (The International Wheat Sequencing Consortium [IWGSC] 2014) using ProteinPilot Software v4.5 (AB SCIEX). Proteins were identified using the following parameters: sample type = iTRAQ 4-plex (peptide-labeled), Cys; alkylation = iodoacetamide; and digestion = trypsin. The MS proteomics data have been deposited at the iProx, which is an official member of ProteomeXchange Consortium, with the data set identifier IPX0001525003/PXD012900. An Unused Protein Score of 1.3 or more (equivalent to 95% confidence level) was used as threshold for a reliable protein identification. A 1.5-fold cutoff and a *P* value < 0.05 was set to identify the significant changes in abundance for the regulated proteins. The

functional annotation and classification of all DEPs were also performed using the KEGG database.

qRT-PCR validation.

Total RNA was extracted using the method described above. First-strand complementary DNAs were synthesized using a PrimeScript first-strand cDNA synthesis kit (TaKaRa). PCR amplification of 18 selected DEGs of the given colonized wheat was performed with an Applied Biosystems 7500 Real-time PCR system (Applied Biosystems) using SYBR AdvantageqPCR Premix (Toyobo). Primers used for qRT-PCR amplification targeting the selected genes are listed in Supplementary Table S7. A fragment of the α -tubulin gene (GenBank accession number U76558) was amplified with specific primers TUBF (5'-ACC GCC AGC TCT TCC ACC CT-3') and TUBR (5'-TCA CTG GGG CAT AGG AGG AA-3') as the internal control for qRT-PCR analysis. A reaction mixture (20 μ l) consisted of 10 μ l of 2 $\times$ SYBR Green PCR Master mix, 0.4 μ l of each primer (10 μ M), 2 μ l of cDNA, and 7.2 μ l of nuclease-free water. qPCR conditions comprised one cycle at 95°C for 10 s, followed by 40 cycles at 95°C for 4 s, 58°C for 15 s, and 72°C for 34 s. Melting curve analysis and electrophoresis were performed to verify the specificity and identity of the qRT-PCR products. In total, nine replicates were used for each sample (three biological replicates $\times$ three technical replicates). Gene expression was evaluated by the 2^{- $\Delta\Delta$ CT} method (Livak and Schmittgen 2001).

Statistical analysis.

The phytohormone data between treatments were subjected to analysis of variance and considered statistically significant at *P* value < 0.05 according to Fisher's least significant difference test. GraphPad Prism software (version 3.02) or R (version 3.5.0) was used to prepare figures.

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AUTHOR-RECOMMENDED INTERNET RESOURCES

IWGSC index: <https://urgi.versailles.inra.fr/download/iwgsc>

Kyoto Encyclopedia of Genes and Genomes database: <https://www.kegg.jp>

Integrated Proteome Resources database: <https://www.iprox.org>

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