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Proteomic Reference Map and Comparative Analysis between *Streptomyces griseus* S4-7 and *wbiE2* Transcription Factor-Mutant Strain

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***Streptomyces griseus* S4-7, a well-characterized keystone taxon among strawberry microbial communities, shows exceptional disease-preventing ability. The whole-genome sequence, functional genes, and bioactive secondary metabolites of the strain have been described in previous studies. However, proteomics studies of not only the S4-7 strain, but also the *Streptomyces* genus as a whole, remain limited to date. Therefore, in the present study, we created a proteomics reference map for *S. griseus* S4-7. Additionally, analysis of differentially expressed proteins was performed against a *wblE*2 mutant, which was deficient in spore chain development and did not express an antifungal activity-regulatory transcription factor. We believe that our data provide a foundation for further in-depth studies of functional keystone taxa of the phytobiome and elucidation of the mechanisms underlying plant-microbe interactions, especially those involving the *Streptomyces* genus.**

Keywords **:** antifungal microbe, keystone taxa, secondary metabolite, spore formation, *whi*-type transcription factor

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Suppressive soils are defined as "soils in which the pathogen does not establish or persist, establishes but causes little or no damage, or establishes and causes disease for a while but thereafter the disease is less important, although the pathogen may persist in the soil" (Weller, 2007). In contrast, the disease in question readily occurs and persists in non-suppressive soils (conducive soils). Currently, many different types of suppressive soils have been reported, including those with suppressiveness against *Fusarium oxsporum*, *Streptomyces scabies*, and *Heterodera avenae* (Weller et al., 2002). Fusarium wilt of strawberry is caused by *F. oxysporum* f. sp. *Fragariae*, which forms three types of spores: microconidia, macroconidia, and chlamydospores. Chlamydospores can persist in a dormant stage in the soil for as long as 30 years, and each of these spores can be spread through running water, farm implements, and machinery (Couteaudier and Alabouvette, 1990). Reliable chemical control of strawberry Fusarium wilt disease is unavailable to date. *Streptomyces griseus* S4-7 has been isolated from a strawberry field and characterized as the suppressive agent in Fusarium wilt in a previous study (Cha et al., 2016). Interestingly, the strain was detected in the strawberry flower as well as in the pollinator body (Kim et al., 2019a). *Streptomyces griseus* S4-7 is unnoted the most of basic information such as antibiotic biosynthesis pathway, regulatory mechanisms (Kim et al., 2019b). Previous proteomic studies of *Streptomyces* spp. focused on membrane proteome analysis and gene mapping for substance analysis. The *wblE2* mutant, deficient in a *whi*-type transcription factor, failed to inhibit the growth of the fungal pathogen or confer plant protection (Cho et al., 2017). In the present study, we aimed to create a proteomic reference map of the S4-7 strain. Proteomic mapping of S4-7 should

provide insights into S4-7 physiology and enable investigation of its potential for antibiotic biosynthesis and biocontrol applications.

Bacterial strains, S4-7 and *wblE2* mutant, were cultured in 25 ml of YEME (yeast extract 3 g, malt extract 3 g, peptone 3 g, glucose 10 g, sucrose 170 g, and agar 20 g per liter). The cells were harvested by centrifugation and resuspended as 10^8 colony-forming units/ml. Then, 1 ml of the bacterial solution was inoculated onto nitrocellulose membranes coated with MS (mannitol soya flour agar: 20 g of mannitol, 20 g of soya flour, and 20 g of agar per 1 liter). The mycelium was scraped from the cellophane discs at three different time points during colony development for further observation: at 24 h, when only vegetative mycelium was observed; at 48 h, when aerial mycelium covered the plates; and at 72 h, when the color of the mycelium surface turned gray because of sporulation (Supplementary Fig. 1).

Proteins were extracted from the S4-7 and Δ*wblE2* strains at three developmental time points (24, 48, and 72 h) using the trichloroacetic acid/acetone/phenol extraction method (Kwon et al., 2014). The extracted proteins were quantified using a 2D-Quant Kit (Amersham Biosciences, Bukinghamshire, UK). Soluble protein (300 μg) was used for two-dimensional gel electrophoresis (2-DE) analysis. For isoelectric focusing, PROTEAN IEF cell (Bio-Rad, Hercules, CA, USA) was used with immobilized pH gradient strips (17 cm, pH 4-7, Bio-Rad). Then, two-dimensional sodium dodecyl sulfate polyacrylamide gel electrophoresis was performed using the Protein Xi-II Cell System

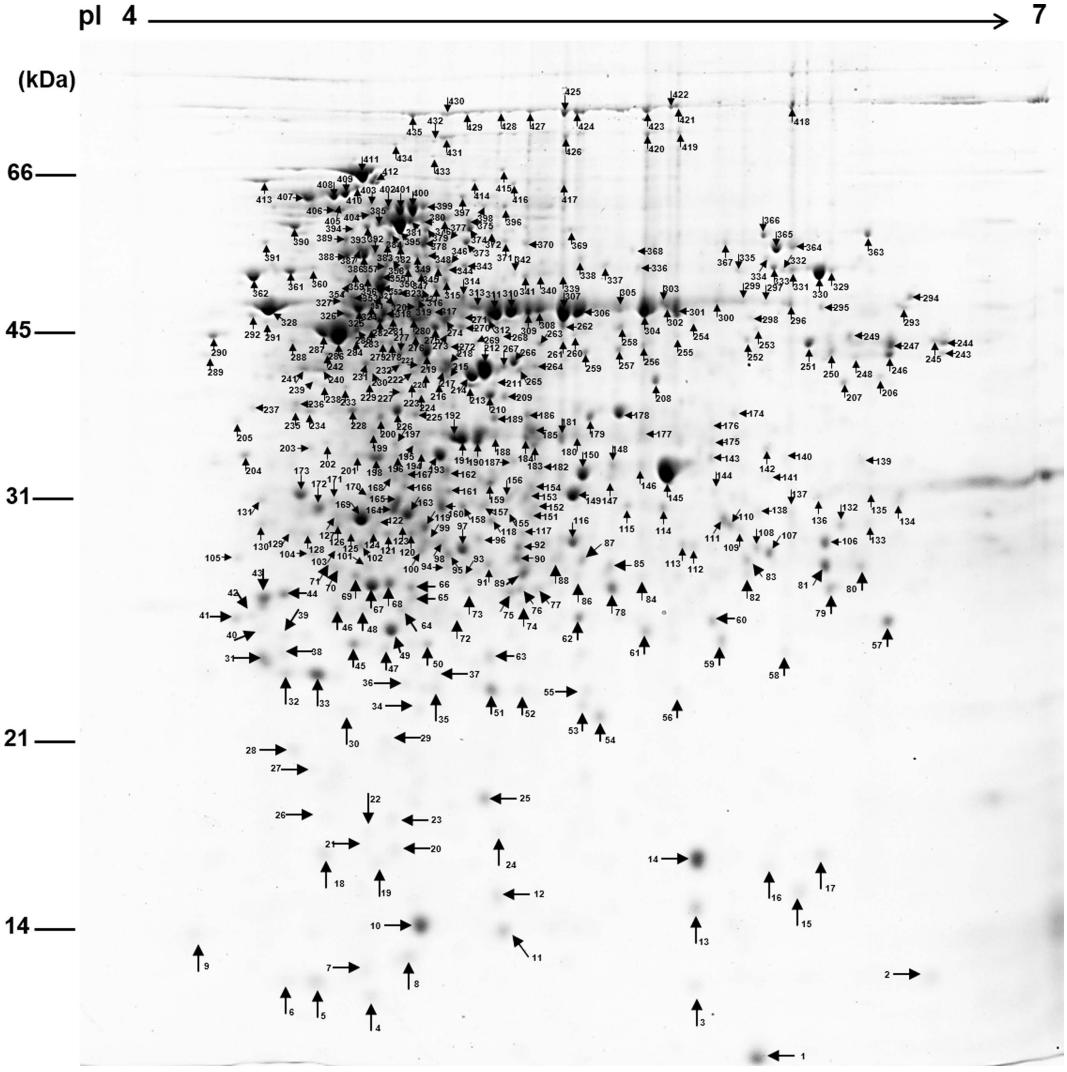

Fig. 1. Protein expression profile of *Streptomyces griseus* S4-7. Proteins loaded onto 17 cm isoelectric focusing strips, pH 4 to 7 linear gradient. The strip was placed on top of 12.5% polyacrylamide gels for sodium dodecyl sulfate polyacrylamide gel electrophoresis. The gel was stained with Coomassie Brilliant Blue R-250.

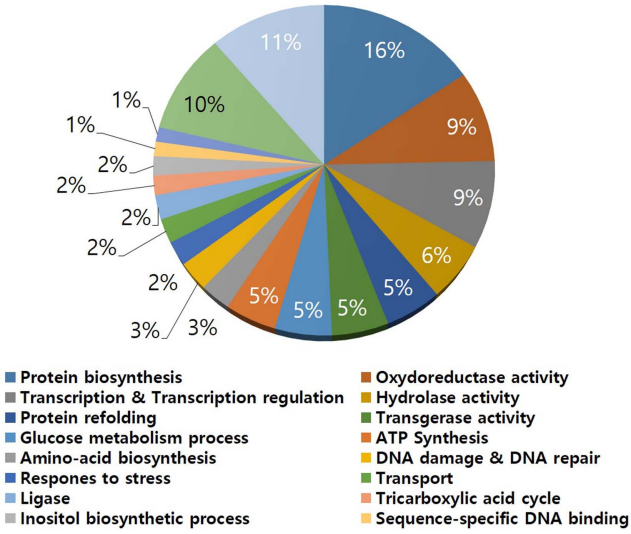

Fig. 2. Functional classification of identified proteins in *Streptomyces griseus* S4-7.

(Bio-Rad). The gels were stained with Colloidal Coomassie Blue G-250 (Candiano et al., 2004). Images were acquired using a GS-800 Imaging Densitometer (Bio-Rad). Abundant proteins were detected with PDQUEST (version 7.2.0, Bio-Rad). Spot densities were normalized to a relative density, and the mean values from triplicates were compared. The cut-off for differential expression was set at 1.5-fold change. To identify abundant proteins, the spots were excised and digested by in-gel tryptic digestion. The cleavage (peptide) solution was loaded onto a matrix-assisted laser desorption inonization-time of flight/mass spectrometry (MALDI-TOF/MS) sample plate and analyzed by an ABI 4800 Plus TOF-TOF mass spectrometer (Applied Biosystems, Foster City, CA, USA). MALDI-TOF MS spectra were queried using the National Center for Biotechnology Information (NCBI) protein database with ProteinPilot (version 3.0, AB Sciex, Framingham, MA, USA) and the MASCOT search engine (version 2.3.02, Matrix Science, London, UK). The statistical significance threshold was set at $P < 0.05$.

In *S. griseus*, a total of 435 protein spots were detected from the 2-DE gel (Fig. 1) and 223 proteins were identified and assigned functions (Supplementary Table 1). Functional distribution of the identified proteins was as follows: protein biosynthesis (16%), oxidoreductase activity (9%), transcription and regulation (9%), hydrolase activity (6%), protein folding (5%), transferase activity (5%), glucose metabolism process (5%), ATP synthesis (5%), amino acid biosynthesis, DNA damage and repair, response to stress,

Table 1. List of difference abundant proteins in the S4-7 and *wblE2* mutant during developmental stages

No.	Protein	ID (UniProt)	MW/pI, theor.	SC (%)	Score[a]	MP	Peptide hit[b]	Fold change S4-7 vs. *wblE2*			Biological function
								24 h	48 h	72 h	
1	10 kDa chaperonin	B1W3U3	10,983/4.81	65	246	5	4	–1.62	+2.45	+1.86	Protein folding
2	50S ribosomal protein L29	S2Y924	8,423/6.59	39	75	4	2	-	+1.58	+1.60	Translation
3	Uncharacterized protein	Q9RJ72	14,528/4.67	45	41	5	0	-	–2.96	–2.54	Unknown
4	Glyoxalase	V6KE74	16,023/4.98	62	48	6	0	-	-	+3.26	Stress response
5	Peptidyl-prolyl cis-trans isomerase	Q53IB1	17,782/6.29	66	218	9	4	-	+2.33	+1.92	Protein folding
6	Transcriptional regulator, CarD family	G0PWJ3	17,818/5.54	62	255	7	5	-	+1.82	+1.65	Transcription
7	GNAT family toxin-antitoxin system, toxin component	D9WA37	20,319/6.05	41	42	4	0	-	+2.05	+1.67	Stress response
8	ATP-dependent Clp protease proteolytic subunit	G2NHF8	21,617/4.58	47	154	9	4	-	-	+2.24	Stress response
9	Single-stranded DNA-binding protein	B4V9L5	20,053/5.29	37	240	7	4	-	+2.47	+1.88	DNA replication
10	Ribosome-recycling factor	G0Q1P2	20,904/5.46	48	444	16	8	-	-	+5.49	Translation
11	Crp/Fnr family transcriptional regulator	A0A087KFI7	51,508/5.23	19	149	11	4	-	–3.35	–3.71	Transcription
12	Crp/Fnr family transcriptional regulator	A0A087KFI7	51,508/5.23	22	339	13	7	-	–6.23	–7.34	Transcription

(Continued)

Table 1. Continued

No.	Protein	ID (UniProt)	MW/pI, theor.	SC (%)	Score[a]	MP	Peptide hit[b]	Fold change S4-7 vs wblE2			Biological function
								24 h	48 h	72 h	
13	50S ribosomal protein L25	W9FNI6	20,450/4.86	23	114	4	2	–2.49	-	-	Translation
14	Proteasome subunit beta	V6UE24	30,176/4.85	54	520	16	8	-	–2.11	–1.92	Protein degradation/ pathogenesis
15	Elongation factor Tu	A6N2C1	36,111/5.01	34	405	9	5	–1.63	–12.34	–10.78	Translation
16	Uracil-DNA glycosylase	W9FK54	25,330/5.71	47	144	9	4	-	+2.65	+3.20	DNA damage /DNA repair
17	Crp/Fnr family transcriptional regulator	A0A087KFI7	51,508/5.23	33	427	14	7	-	–3.49	–4.22	Transcription
18	Chlorite dismutase	W9FXT2	27845/5.90	68	491	16	8	-	+1.96	-	Stress response
19	Lipoprotein	M3FS09	30955/5.06	31	38	6	0	+2.44	-	-	Transport/Virulence
20	Electron transfer flavoprotein subunit alpha	A0A087JXW9	32481/4.97	44	666	8	8	-	+3.13	+1.95	Transport
21	Proteasome subunit alpha	G0Q7E7	29503/4.83	35	222	9	4	–3.97	-	–2.68	Protein degradation
22	Methyltransferase	A0A087K8E5	32599/6.37	70	619	19	7	-	+4.72	+3.51	Stress response
23	Cysteine desulfurase	A0A087KB50	27328/5.25	68	303	14	5	-	+2.96	+2.04	Transport
24	Crp/Fnr family transcriptional regulator	A0A087KFI7	51508/5.23	33	533	14	7	-	-	–1.87	Transcription
25	Phosphotransferase	F2R313	26746/4.78	38	54	7	0	-	+3.40	+2.82	Transport
26	Phosphoribosylaminoimidazole-succinocarboxamide synthase	W9FWD8	33816/4.75	51	235	11	5	-	–3.29	–2.13	Purine biosynthesis
27	Sulfurtransferase	A0A087JZZ5	31,629/4.75	67	417	17	8	+1.98	+3.77	-	Stress response
28	Cellulose-binding protein	A0A087KFW0	34,734/5.28	62	333	21	7	-	+4.44	+2.60	Cell wall biogenesis/ degradation
29	Polyprenyl synthetase	W9FVB1	36,453/5.29	51	121	11	2	-	+2.31	+1.74	Isoprene biosynthesis
30	UDP-glucose 4-epimerase	W9FYS0	34,585/5.33	54	175	13	3	-	-	+4.38	Carbohydrate metabolism
31	ATP-binding protein	A0A087KCQ4	36,214/5.10	28	88	9	2	-	+2.96	+1.84	Transport
32	Peptide chain release factor 2	B1VV08	40,843/4.70	54	284	15	6	-	+3.30	+2.19	Protein biosynthesis/ translation
33	Argininosuccinate synthase	B1W3B3	43,579/4.94	35	250	13	4	-	+1.99	+1.64	Arginine biosynthesis
34	Acetylornithine transaminase	G0PPG1	45,532/5.79	35	209	11	4	-	–2.40	-	Arginine/lysine biosynthesis
35	Citrate synthase	G0Q9J5	48,303/5.71	65	702	31	10	-	–1.74	-	Carbohydrate metabolism
36	Chorismate synthase	B1W450	41,587/5.78	38	261	12	5	-	+1.72	+2.15	Chorismate biosynthetic process
37	Glycogen phosphorylase	B5GKX7	56,486/5.67	20	149	10	3	-	–3.38	-	Carbohydrate metabolism
38	Bifunctional protein GlmU	B1VUI7	49,939/5.72	41	218	14	3	-	+2.24	+2.11	LPS lipid A biosynthesis
39	Trigger factor	B1VXA5	51,286/4.33	45	297	21	6	-	+3.42	+3.86	Cell cycle/cell division/
40	30S ribosomal protein S1	A0A069JPK5	55,007/4.55	50	699	24	8	-	+7.43	+6.70	Translation
41	30S ribosomal protein S1	B1W0V3	55,094/4.55	54	729	28	10	-	+9.54	+5.67	Translation

MW, molecular weight; SC, the percentage of sequence coverage; MP, the number of matched peptides; MS, mass spectrometry; MS/MS, tandem MS.

[a]Protein score based on the combined MS and MS/MS spectra.

[b]Peptide hits as the unique number of MS/MS spectra that matched the trypsin peptide.

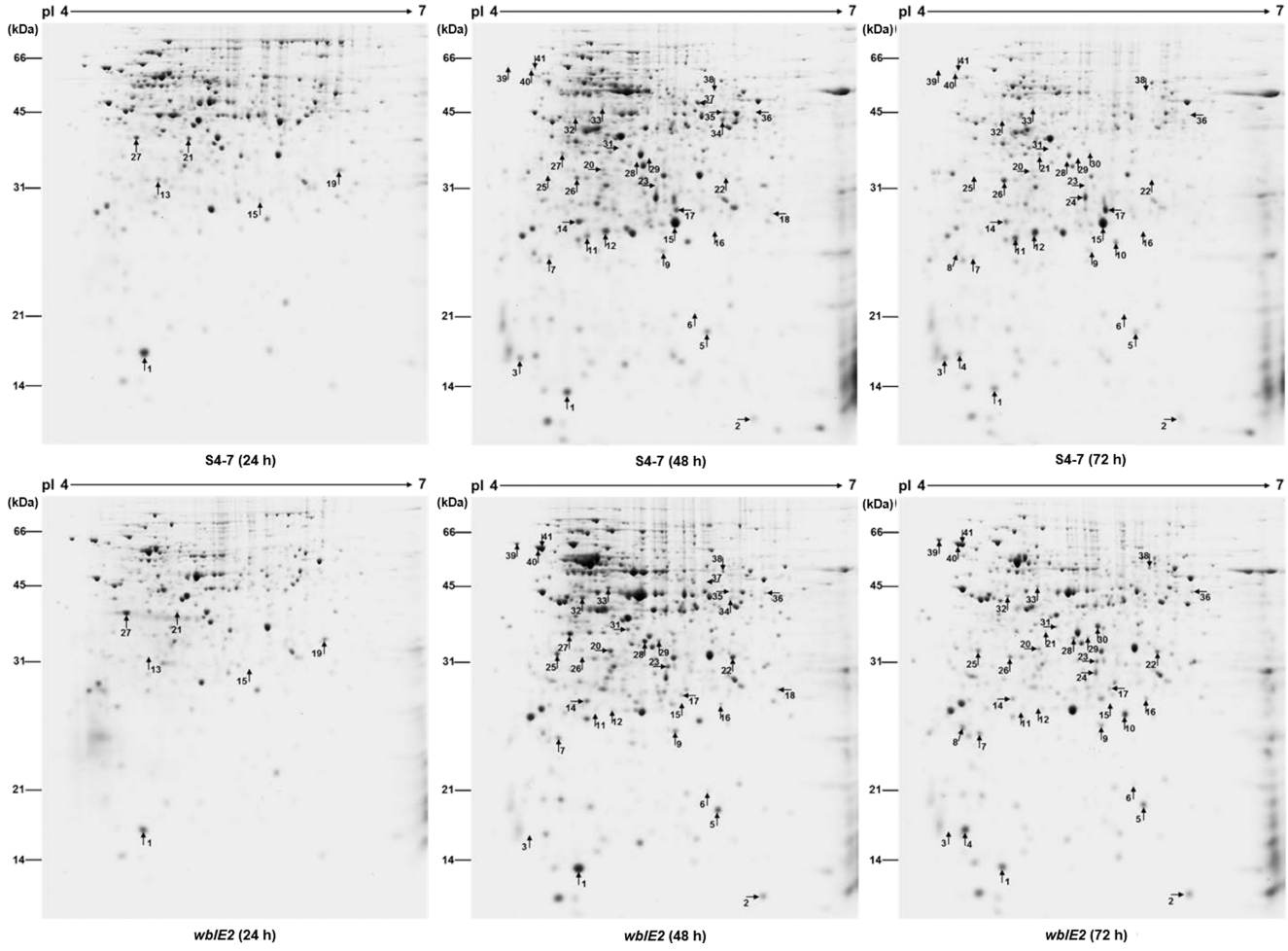

Fig. 3. Representative two-dimensional gel electrophoresis image of the S4-7 and *wblE2* mutant at different developmental stages. A total of 300 μg of soluble protein was loaded and separated using an immobilized pH gradient strip (17 cm, pH 4-7) and 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis. The numbers on the gels showed differentially expressed proteins between the S4-7 and the *whiE2* mutant.

transport, ligase, tricarboxylic acid cycle and inositol biosynthesis process (2% each), hypothetical protein (11%), and uncharacterized protein (11%) (Fig. 2). The data indicated that the strain used energy sources mainly for primary metabolism, and that the strain required large amounts of amino acids and related metabolic processes for development and growth under laboratory culture conditions.

In total, 41 proteins were detected as difference in abundance proteins in the wild-type S4-7 and Δ*wblE2* strains (Table 1, Fig. 3). All the detected proteins were identified as proteins of *Streptomyces* origin after database searching. At 24 h, only six proteins were showed a difference in abundance. In contrast, at 48 h and 72 h, 33 and 34 proteins, respectively, were identified as significantly regulated by the *whi* transcription factor. Among the difference abundant proteins, 13 were consistently less abundance in the mutant strain. Among the carbohydrate metabolism proteins, two were less abundant proteins and the other two were more abundant proteins. Most of the identified stress response, transport, DNA repair, and amino acid metabolism proteins were more abundant in the Δ*wblE2* strain. Interestingly, five transcription-related proteins were detected as difference in abundance, and four of them were significantly less abundant proteins. The four less abundant transcription-related proteins were identified as the cyclic AMP receptor protein/fumarate-nitrate-reductase (*crp*/*fnr*) family transcriptional regulator, which is the key transcription factor in antibiotic production in Actinomycetes. The function of the *crp*/*fnr* transcription factor, similar to that of *whi* transcription factors, is known to involve regulation of colony development and spore germination in *Streptomyces* (Derouaux et al., 2004). Recently, a *crp*/*fnr* family

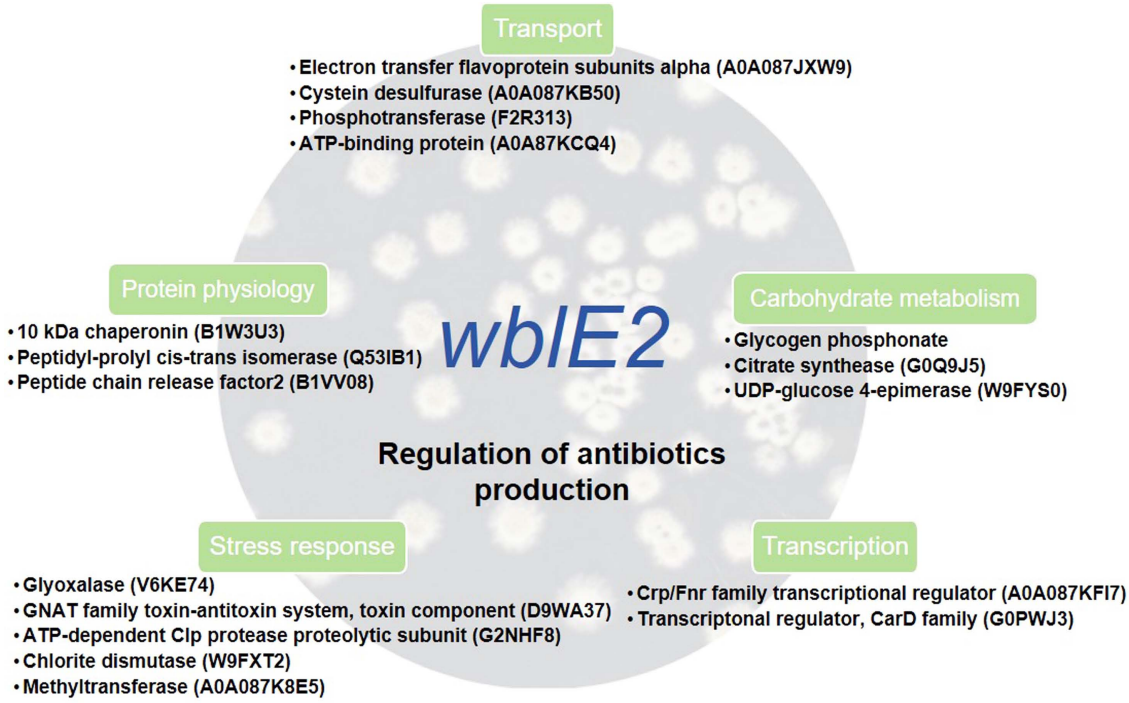

Fig. 4. A possible model for the mechanism of action of *whi* transcription factor in antibiotic production and regulation of *Streptomyces griseus* S4-7.

transcriptional regulator was described as a global regulator of antibiotic production in *Streptomyces* (Gao et al., 2012). In our proteomic analyses, *crp*/*fnr* genes were significantly less abundant proteins in the Δ*whi* strain relative to the wild-type strain (Table 1). These results indicate that (1) the *whi* transcription factor regulates antibiotic production associated with the plant-protective ability of the S4-7 strain in the strawberry rhizosphere; and (2) this transcription factor may act upstream of the *crp*/*fnr* genes during colony development and secondary metabolite production. A typical phenotype of the Δ*crp* comprises reduced colony development and germination, but accelerated sporulation (Derouaux et al., 2004; Gao et al., 2012). By contrast, morphological characteristics of the Δ*wblE*2 included decreased spore and aerial hypha development.

In conclusion, the *S. griseus* S4-7 strain has exceptional ability to suppress the growth of *F. oxysporum* f. sp. *fragariae*; however, the underlying antibiotic biosynthesis pathways and regulatory mechanisms remain to be elucidated. In this study, proteomics mapping of *S. griseus* S4-7 proteins provided novel insights into these aspects (Fig. 4). All 435 proteins of the strain were observed; among them, 223 proteins were identified. Additional study of these functions is warranted. The present findings establish a foundation to explore the potential of the *S. griseus* S4-7 strain as a new biocontrol agent.

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Electronic Supplementary Material

Supplementary materials are available at The Plant Pathology Journal website(http://www.ppjonline.org/).

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