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Identification of Genes Associated with Fumonisin Biosynthesis in *Fusarium verticillioides via* Proteomics and Quantitative Real-Time PCR

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In this study, we used functional genomic strategies, proteomics and quantitative real-time (qRT)-PCR, to advance our understanding of genes associated with fumonisin production in the fungus *Fusarium verticillioides*. Earlier studies have demonstrated that deletion of the *FCC1* gene, which encodes a C-type cyclin, leads to a drastic reduction in fumonisin production and conidiation in the mutant strain (FT536). The premise of our research was that comparative analysis of *F. verticillioides* wild-type and FT536 proteomes will reveal putative proteins, and ultimately corresponding genes, that are important for fumonisin biosynthesis. We isolated proteins that were significantly upregulated in either the wild type or FT536 *via* two-dimensional polyacrylamide gel electrophoresis, and subsequently obtained sequences by mass spectrometry. Homologs of identified proteins, *e.g.*, carboxypeptidase, laccase, and nitrogen metabolite repression protein, are known to have functions involved in fungal secondary metabolism and development. We also identified gene sequences corresponding to the selected proteins and investigated their transcriptional profiles *via* quantitative real-time (qRT)-PCR in order to identify genes that show concomitant expression patterns during fumonisin biosynthesis. These genes can be selected as targets for functional analysis to further verify their roles in FB_1 biosynthesis.

Keywords: Fumonisin regulation, fungal development, proteomics, quantitative real-time PCR, gene discovery

Fusarium verticillioides (Sacc.) Nirenberg (teleomorph *Gibberella moniliformis* Wineland) is a plant pathogenic fungus of maize that produces fumonisins, a group of polyketide-derived mycotoxins, on maize and maize-based products [23, 24]. Fumonisin-contaminated food and feeds have been linked to a variety of human and animal illnesses, including leukoencephalomalacia, esophageal cancer, and neural tube defects [8, 22, 38]. Studies have shown that these toxic effects are due to the fact that fumonisins can inhibit ceramide synthase and ultimately disrupt sphingolipid metabolism in animals [21, 36]. Significantly, a possible link between fumonisin-contaminated food and human illnesses, such as esophageal cancer and neural tube defects, has raised food safety and public health concerns [22, 38]. Owing to these concerns, a guideline for fumonisin levels in food and feeds was implemented by the U.S. Food and Drug Administration in 2001 [25]. In particular, fumonisin B_1 (FB_1), which is the predominant fumonisin found in nature, has been analyzed and closely monitored in foods and feeds.

While researchers have invested substantial efforts to understand the chemistry, toxicology, and biology of fumonisin since its discovery, the current research focus is centered around the molecular mechanism associated with fumonisin biosynthesis in *F. verticillioides*. Proctor *et al.* [27] isolated and characterized *FUM1*, a gene encoding a polyketide synthase in *F. verticillioide*. *FUM1* was the first gene identified in what is now determined as the fumonisin biosynthetic (*FUM*) gene cluster, which contains at least 15 coregulated genes [28]. It is interesting to note that a pathway-specific regulatory gene has not been characterized in the *FUM* gene cluster to date, and that the regulatory mechanisms involved in fumonisin biosynthesis are considered complex [32]. A number of genes regulating fumonisin biosynthesis have been isolated and characterized, but none of these genes are present in the *FUM* gene cluster [32]. Molecular characterization of these regulatory genes demonstrated that these genes are critical for fumonisin biosynthesis in *F. verticillioides* [4, 5, 35]. In addition, the fact that physiological and nutritional conditions, notably acidic pH and nitrogen stress, favor or perhaps trigger fumonisin biosynthesis suggests that the mechanism associated with fumonisin biosynthesis a complex biological process [4, 34].

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A variety of molecular genetic approaches including restriction enzyme-mediated integration (REMI) mutagenesis, degenerate PCR cloning, and suppressive subtraction hybridization (SSH) were employed to isolate genes associated with the FB_1 regulatory mechanism [4, 35]. To date, regulatory genes such as *PAC1*, *FCC1*, *ZFR1*, and *GBP1* were identified as genes associated with FB_1 biosynthesis [4, 5, 31, 35]. In particular, *FCC1* has been identified as a gene regulating early signal transduction events that trigger FB_1 biosynthesis in *F. verticillioides* [35]. To further our understanding of this complex regulatory mechanism, genomics strategies have been applied to accrue more comprehensive information. Pirttilä *et al.* [26] constructed microarrays with expressed sequenced tags (ESTs) from *F. verticillioides* wild type and the *FCC1* deletion mutant (FT536) and identified genes that are expressed concomitantly with fumonisin production. The study identified 19 genes displaying expression profiles similar to the *FUM* genes. Furthermore, Brown *et al.* [1] reported generating over 87,000 ESTs from *F. verticillioides*. Analysis of this extensive collection of ESTs, which is estimated to represent over 80% of the expressed genes in the fungus, revealed eight tentative consensus (TC) sequences that are likely to regulate fumonisin biosynthesis. Further functional characterization of these putative regulatory genes will provide additional insight into fumonisin regulation in *F. verticillioides*.

However, although transcriptional profiling is a commonly accepted strategy for identifying genes associated with certain biological processes, it is important not to discount its limitations. The mRNA levels of a certain gene represent short-term changes in the expression and may influence downstream gene regulation and ultimately cellular function. On the other hand, mRNA abundance may not be indicative of a gene's regulatory or metabolic potential. In some instances, pathway regulations occur at the post-transcriptional level, and therefore the activity of the final gene product, the protein, may provide better evaluation of a gene's functional role. Thus, to complement these approaches and isolate genes with potential involvement in FB_1 biosynthesis, we analyzed the proteomic changes that are due to *FCC1* gene mutation in *F. verticillioides*. Deletion of *FCC1* in *F. verticillioides* resulted in drastic reduction of fumonisin biosynthesis and conidia production [35]. In addition, one of the key reasons we selected the FT536 for proteome analysis, in comparison with the wild-type strain, is the availability of the gene expression profile in two strains during FB_1 biosynthesis and fungal development [26].

The objective of this study was to identify proteins that are differentially regulated in either the wild type or FT536. Our premise is that these differentially expressed proteins are associated with fumonisin biosynthesis or conidiation in *F. verticillioides*. To perform direct comparative analysis of the gene expression profile versus the proteome profile, we incubated the wild-type and FT536 strains in the same culture condition, BSAL medium that is conducive to fumonisin biosynthesis, as described previously [26]. We also isolated corresponding gene sequences and investigated their transcriptional profiles *via* quantitative real-time (qRT)-PCR in order to identify protein-gene pairs that show concomitant expression patterns during FB_1 biosynthesis.

MATERIALS AND METHODS

Fungi and Culture Media

The wild-type *F. verticillioides* strain 7600 (also designated M-3125; Fungal Genetics Stock Center, Kansas City, KS) and the fumonisin-deficient mutant strain FT536 [35] were stored in 30% glycerol at −80°C. The fungi were grown on V8 agar (200 ml V8 juice, 3 g $CaCO_3$, 20 g agar per liter) for inoculum. For protein and total RNA extraction, the fungal strains were grown in BSAL medium (22 mM KH_2PO_4, 2.5 mM $MgSO_4$, 85 mM NaCl, 117 mM sucrose, and 1 g/l bovine serum albumin) adjusted to pH 6 [26]. An Erlenmyer flask containing 100 ml of BSAL medium was inoculated with 1×10^7 conidia and incubated on a rotary shaker (150 rpm) at 24°C for 7 days before harvesting fungal tissue. The culture filtrates were analyzed for FB_1 using high-performance liquid chromatography (HPLC) [34].

Proteins Sample Preparation

Protein extracts were prepared by grinding *F. verticillioides* tissues with grinding buffer (50 mM NaCl, 1% SDS, 10 mM Tris [pH 7.4], and 1 mM EDTA) in liquid nitrogen. The homogenized fungal tissues were centrifuged, and the recovered supernatants were mixed with cracking buffer (3.8 g Tris [pH 6.8], 5 g SDS, 5 ml β-mercaptoethanol, 5 mg bromophenol blue, and 50 ml glycerol, per liter). The extracted protein suspension was heated at 100°C for 3 min prior to SDS-polyacrylamide gel electrophoresis (PAGE). Protein concentration was determined by the Bradford method (Bio-Rad, Hercules, CA, U.S.A.). Protein samples (20 mg) were separated in 7.5% and 18% polyacrylamide gels, and subsequently stained with Coomassie brilliant blue.

Two-Dimensional Polyacrylamide Gel Electrophoresis (2D-PAGE)

All the reagents used for 2D-PAGE were of analytical grade and purchased from Amersham Pharmacia Biotech Korea Ltd (Seoul, Korea) unless described otherwise. Sequencing grade trypsin and α-cyano-4-hydroxycinnamic acid was purchased from Promega (Madison, WI, U.S.A.) and Sigma (St. Louis, MO, U.S.A.), respectively. Trifluoroacetic acid (TFA) and acetonitrile were purchased from Merck (Piscataway, NJ, U.S.A.). The protein sample supernatants were mixed with an equal volume of 20% trichloroacetic acid (TCA), incubated for 30 min on ice, and centrifuged at 16,000 rpm at 4°C for 15 min. The supernatants were carefully removed, and the pellets were mixed with cold acetone (300 l) and centrifuged for 5 min at 4°C. The pellet was dried and then resuspended in lysis buffer (7 M urea, 2 M thiourea, 40 mM DTT, 4% CHAPS). 2D-PAGE was performed by vertical electrophoresis, IPGPhor was used for the first-dimensional isoelectric focusing with immobiline DryStrip (24 cm, pH 4–7), and the Ettan DALT system was used for the second-dimensional SDS-PAGE (11% gel). The protein

samples (350 μg) were loaded to the IPG strips (immobilized pH 4–7 linear gradient) by cup loading with the use of IPGphor (24 cm). Isoelectric focusing was carried out successively at 500 V for 1 h, 1,000 V for 1 h, and 8,000 V for 5 h. After isoelectric focusing, strips were incubated with equilibration buffer 1 (50 mM Tris-HCl [pH 8.8], 6 M urea, 30% glycerol, 2% SDS, BPB, and 2% DTT) and buffer 2 (50 mM Tris-HCl [pH 8.8], 6 M urea, 30% glycerol, 2% SDS, BPB, 2% DTT, 2.5% iodoacetamide) for 15 min each. The equilibrated strip was placed on a polyacrylamide gradient slab gel (10–15% gradient). Separation was continued at 20 mA/gel in running buffer (25 mM Tris [pH 8.8], 198 mM glycine, and 0.1% SDS) until the BPB reached the bottom of the gel. The gel was then treated with fixing solution (40% methanol, 10% acetic acid) for 1 h. After fixing, the gel was treated with rehydration solution (30% ethanol) for 30 min, twice, and with sensitizing solution (0.02% sodium thiosulfate) for 1 min, before washing with deionized water. Subsequently, the gel was treated with $AgNO_3$ solution (0.2% $AgNO_3$, 0.02% HCOH) for 30 min, washed with deionized water, and was incubated with developing solution (3% sodium carbonate, 0.05% HCOH) for 3–5 min. Finally, stop solution (0.5% glycine) was added.

Image Analysis and Mass Spectrometry (MS)

The silver-stained gels were scanned with a densitometer 800 (Bio-Rad). Two protein spots that showed similar expression levels in the two gels were identified as control proteins (Fig. 1). The digitized image was analyzed using PDQUEST software (V. 6.1, Bio-Rad). Differentially expressed (greater than 5×) proteins were selected for further mass spectrometry analyses. Proteins were identified by MALDI-TOF MS [11] and QTOF-MS/MS. Selected protein spots were cut from gels with a spot cutter (Bio-Rad), and the excised gel spots were destained with 100 μl of destaining solution (30 mM potassium ferricyanide [Sigma], in 100 mM sodium thiosulfate [Merck]) with shaking for 5 min. After the destaining, the gel spots were incubated with 200 mM ammonium bicarbonate (Sigma) for 20 min. The gel pieces were dried in a speed vacuum concentrator for 5 min and then rehydrated with 20 μl of 50 mM ammonium bicarbonate containing 0.2 μg of modified trypsin (Promega) for 45 min on ice. After removal of the solution, 30 μl of 50 mM ammonium bicarbonate was added and the digestion was performed overnight at 37°C. The peptides were desalted and concentrated using C18 nanoscale (porus C18) columns (In2Gen, Seoul, Korea).

For analysis by MALDI-TOF MS (peptide-mass fingerprinting method), the peptides were eluted with 0.8 ml of matrix solution (70% acetonitrile [Merck], 0.1% TFA [Merck], and 10 mg/ml alpha-cyano-4-hydroxycinnamic acid [Sigma]), and were spotted onto a stainless-steel target plate. Masses of peptides were determined using MALDI-TOF MS (Model M@LDI-R; Micromass, Manchester, U.K.). Calibration was performed using an internal mass of trypsin autodigestion product (*m/z* 2,211.105). For analyses by QTOF-MS/MS, 15 μl of the peptide solutions from the digestion supernatant were diluted with 30 μl of 5% formic acid, loaded onto the column, and washed with 30 μl of 5% formic acid. Peptides were eluted with 2 μl of methanol/H_2O/formic acid (50/49/1, v/v/v) directly into a precoated borosilicate nanoelectrospray needle (EconoTip; New Objective, U.S.A.). MS/MS of peptides generated by in-gel digestion was performed by nano-ESI on a Q-TOF2 mass spectrometer (Micromass, Manchester, U.K.). The source temperature was 80°C. A potential of 1 kV was applied to the precoated borosilicate nanoelectrospray needles in the ion source combined with a nitrogen

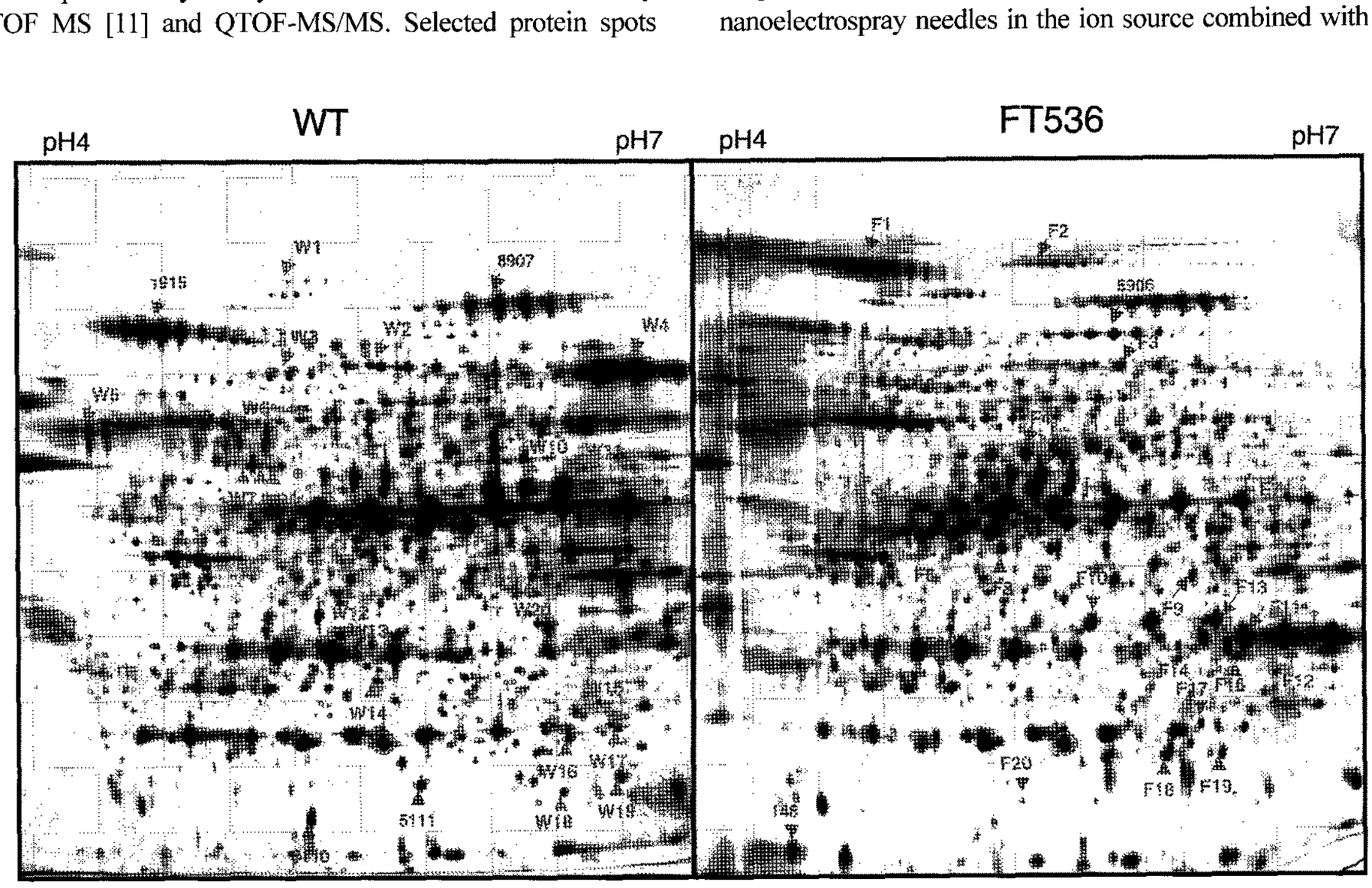

Fig. 1. Silver-stained protein expression profile of *F. verticillioides* wild-type and FT536 strains.

Both strains were grown in culture medium conducive to FB_1 biosynthesis, for 7 days, and total proteins from fungal mycelia were extracted. Protein samples (350 μg) were applied to two-dimensional polyacrylamide gel electrophoresis for separation. Here, a representative figure from three technical and two biological replicates is shown for wild type and FT536. Arrows (with designation) in the figure indicate proteins that are specifically produced or up regulated in WT or FT536 protein samples. The control protein (glyceraldehyde 3 phosphate dehydrogenase) is circled in WT and FT536 panels.

back-pressure of 0–5 psi to produce a stable flow rate (10–30 nl/min). The mass spectrometer operated in an automatic data-dependent MS/MS to collect ion signals from the eluted peptides. In this mode, the most abundant peptide ion peak with a double- or triple-charged ion in a full-scan mass spectrum (*m/z* 400–1,500) was selected as the precursor ion. Finally, an MS/MS spectrum was recorded to confirm the sequence of the precursor ion using collision-induced dissociation (CID) with a relative collision energy dependant on molecular weight. The cone voltage was 40 V. The quadrupole analyzer was used to select precursor ions for fragmentation in the hexapole collision cell. The collision gas was Ar at a pressure of $6–7\times10^{-5}$ mbar and the collision energy was 20–30 V. Product ions were analyzed using an orthogonal TOF analyzer, fitted with a reflector, a microchannel plate detector, and a time-to-digital converter. The data were processed using a Mass Lynx Windows NT PC system.

To identify the protein, protein masses from MALDI-TOF MS were matched with the theoretical molecular weight of peptides for proteins in the NCBI nr database using MASCOT software. Additionally, all MS/MS spectra recorded on tryptic peptides derived from the spots were searched against protein sequences from NCBI nr and the *F. verticillioides* Gene Index (http://compbio.dfci. harvard.edu/tgi/), and the *F. verticillioides* genome database (http://www.broad.mit.edu/annotation/genome/fusarium_verticillioides/Home.html) using the MASCOT search program (http://www.matrixscience.com).

Expression Profiles of Genes Corresponding to the Expressed Proteins

Total RNA for qRT-PCR was prepared with Trizol reagent (Invitrogen, Carlsbad, CA, U.S.A.) or with an RNeasy plant mini kit (Qiagen, Valencia, CA, U.S.A.) as per the manufacturers' protocols. All qRT-PCR reactions were performed in a Cepheid Smart Cycler System (Cepheid, Sunnyvale, CA, U.S.A.) with a QuantiTect SYBR Green RT-PCR kit (Qiagen). All primers used in this experiment are listed in Table 1. Amplification and quantification were performed as described by Sagaram *et al.* [31]. Briefly, qRT-PCR reactions were carried out with 30 min of reverse transcription at 50°C followed by 15 min of pre-denaturation at 95°C and 35 cycles of 15 s of denaturation at 95°C, 30 s of annealing at 55°C, and 30 s of extension at 72°C. All qRT-PCR were technically repeated three times. The gene expression was calibrated using and $2^{-\Delta\Delta Ct}$ method [19] with the β-tubulin gene (*TUB2*) (GenBank U27303) expression as a reference. The range of expression was calibrated using $2^{-\Delta\Delta Ct-s}$-$2^{-\Delta\Delta Ct+s}$, where s is the standard deviation of the ΔCt value (Ct=Threshold Cycle).

RESULTS

Analysis of Differentially Expressed Proteins in the *F. verticillioides* Wild-Type and FT536 2D-PAGE

Protein was extracted from 7-day-old *F. verticillioides* wild-type and FT536 mycelia grown in BSAL, a medium conducive to FB_1 biosynthesis [35], and were prepared for 2D-PAGE. The protein extracts from three independent biological replications were resolved on 1D SDS-PAGE to visualize a reproducible protein separation pattern before proceeding to 2D-PAGE analysis (data not shown). In addition, HPLC analysis verified FB_1 production in the wild-type culture but not in the FT536 culture, in accordance with previously published data [35]. We selected two biological replications and generated three reproducible 2D-PAGE displaying the differentially expressed proteins in two fungal strains per biological replication (data not shown). Approximately 1,150 protein spots were separated in the pH 4–7 range and their images were digitized and analyzed using PDQUEST software (Fig. 1). We performed quantitative comparison of the proteins that are uniquely and differentially regulated in the wild-type and FT536 strains. Based on the results from the 2D-PAGE, we selected

Table 1. Primers used in this study.

Protein	Forward primer sequence (5'-3')	Reverse primer sequence (5'-3')
W3	GGA CTA TCT CCA GGA GAT GAA GAC	GCT CAC AGA CCT TCC AAG C
W4	CCT GTG GAT TGG AAG AAG ACT GG	CCA TAT TTC CAC GAG AAC GGA CC
W10	GAC CTT GCT TCC AGC TGA AGC	CCT TGG TCT TCG AGC TGA GC
W19	CAT CAA GTA CGC CCT CGA G	CAG CCT TGT TGA CGA TCT CC
W20	GTC ACG GCG TGT TGA ATG G	CGA TAT TGG CAC CCT GGT CC
F1	CTG CAC CTT ATG ATG CCA CC	GTG ACG ATC TGA AGC CAG TGG
F8	CTG TCA AGG ACG CTC ACA CTG	CTA CCG TCT TCA TTC TTG CGA ATC G
F10	CTC CCA AGC CTT ACC ATA TTC TAC TC	GCA TCA AAG TTT GCG CCA GC
F11	CTC CTT CGT CTC GTC AAC GG	GAG ATG CTT GAT CAC TCT GCG AC
F15	CGA TTC AAC TCC ACC AAG TCC	GAA CTC CTT GAG CTG AGT CAT GG
F19	GTG CCC CAG TTA ATG ACC TCC	GAC AAG AAC TCT GGC ACT CTG
W1915	GGT ATC AAT GCA CTC CCA ACA ACG TC	CCT CCT CAC TCA TTC TTC CAC C
W3110	CGA TCA CGA ATC AGC GCT C	CCA TAC TTC TCG ACA AGG TTA TAG ACC
W5111	GAG AAG AGC CCC TAT GTC AGC	GTG CTC CAG GGG TGA ATA GC
F148	GCA CCA GAC TTC TCG ATG C	CGA CAA GAT CAA GGC CAA GG
F2719	CAA GGT CGA GGA GAA GAA GTA CG	GCG TTG CAG GAC TTG AGC TC
F5611	GGA CAA GAT CGC TGA TCA GG	CTC GAG AGC CTT CTC GTA GTG
F5906	CCT TAT TGC CAT CTG GGA CGA C	CTG TTT GAT ATC GTC GGC CTT GAG

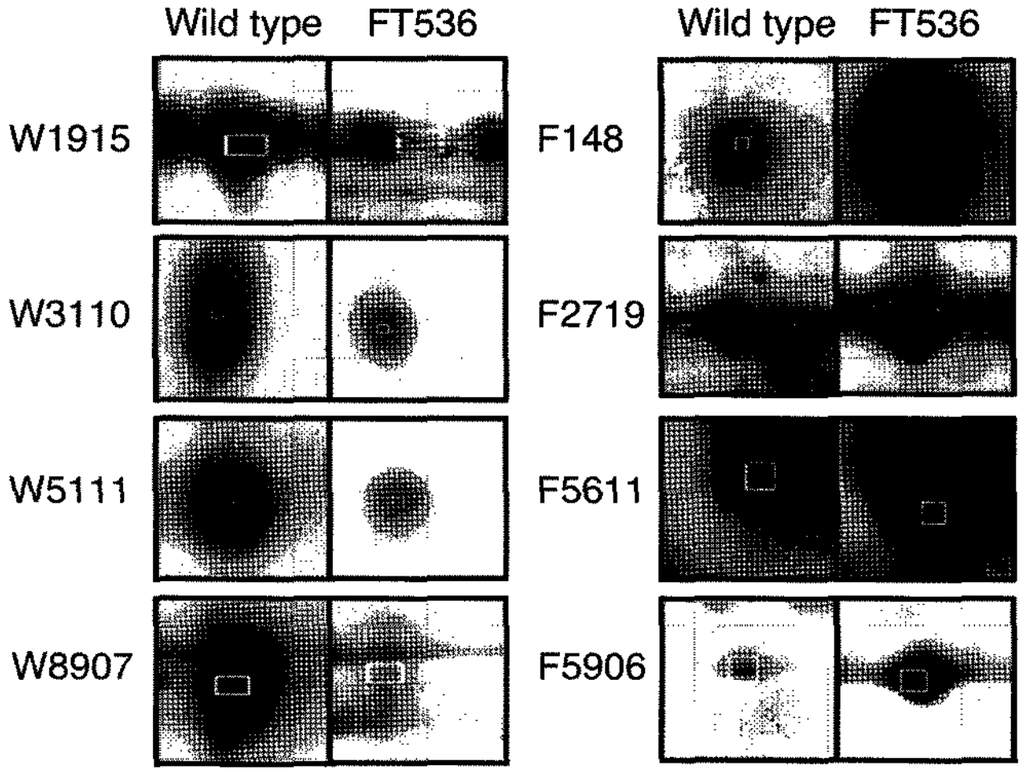

Fig. 2. Two-dimenstional gel electrophoresis close-up image of differentially expressed proteins in *F. verticillioides* wild-type and FT536 strains.

The silver-stained gel was scanned and the digitized images were compared using PDQUEST software. Proteins that show greater than 5-fold increase were selected. Proteins W1915, W3110, W5111, and W8907 are proteins upregulated in the wild-type strain, and proteins F148, F2719, F5611, and F5906 are proteins upregulated in the FT536 strain.

48 proteins for MS analysis. Twenty of these proteins were expressed only in the wild type, twenty were expressed only in FT536, and eight exhibited a difference (greater than 5×) in expression between the two strains (Fig. 2).

Functional Classification of Differentially Expressed Proteins Identified in the Wild-Type and FT536 2D-PAGE

A total of forty-eight differentially expressed proteins were initially analyzed *via* MALDI-TOF MS. Twenty-nine proteins that did not provide significant database matches were further analyzed *via* QTOF-MS. In the end, thirty-seven out of forty-eight selected proteins resulted in interpretable MS data. Subsequently, the best homologs for each protein were identified *via* searches against protein sequences from NCBI nr, the *F. verticillioides* Gene Index at the Dana Farber Cancer Institute, and the *F. verticillioides* genome database at the Broad Institute using the MASCOT MS/MS Ion Search. The protein sequences were grouped into three categories: proteins expressed only in the wild type, only in FT536, and differentially in wild type and FT536.

Table 2. Proteins uniquely expressed in wild-type protein samples.

Spot no.	MS method	*F. verticillioides* locus match[a]	Putative function[b]	Mol. mass[c]	pI[c]	Mascot score[d]	Sequence coverage[e]
W1	Q-TOF	No match	–	–	–	–	–
W2	MALDI	FVEG_05069.3	Pyruvate decarboxylase	63.541	5.60	100	31
W3	Q-TOF	FVEG_04358.3	Alpha-Glucosidase	36.867	7.46	208	30
W4	Q-TOF	FVEG_11127.3	Probable serine-type carboxypeptidase precursor	27.298	8.66	79	32
W5	Q-TOF	FVEG_14045.3	Carboxypeptidase Y precursor	62.181	5.45	39	3
W6	Q-TOF	FVEG_06027.3	Putative peptidase	41.789	5.28	52	3
W7	MALDI	No match	–	–	–	–	–
W8	MALDI	FVEG_11605.3	Saccharopine dehydrogenase	42.424	5.09	75	19
W9	MALDI	No match	–	–	–	–	–
W10	Q-TOF	FVEG_12529.3	*O*-Acetylhomoserine	28.430	6.21	122	22
W11	Q-TOF	FVEG_12529.3	*O*-Acetylhomoserine	28.430	6.21	122	22
W12	MALDI	FVEG_04128.3	Elongation factor 2	91.849	6.45	88	15
W13	MALDI	FVEG_02960.3	Proteasome subunit alpha type 6	27.933	5.69	80	32
W14	Q-TOF	No sequence	–	–	–	–	–
W15	Q-TOF	FVEG_12529.3	*O*-Acetylhomoserine	33.636	6.70	69	9
W16	Q-TOF	No sequence	–	–	–	–	–
W17	MALDI	No match	–	–	–	–	–
W18	Q-TOF	No sequence	–	–	–	–	–
W19	Q-TOF	FVEG_10153.3	Phosphoglycerate kinase	18.586	6.95	33	7
W20	Q-TOF	FVEG_03355.3	Chaperone protein hchA	33.272	5.21	113	8

[a]Gene locus according to the *F. verticillioides* genome database at the Broad Institute of MIT and Harvard (http://www.broad.mit.edu/annotation/genome/fusarium_verticillioides/Home.html).

[b]Descriptions of putative function were obtained from the *F. verticillioides* genome database at the Broad Institute of MIT and Harvard (http://www.broad.mit.edu/annotation/genome/fusarium_verticillioides/Home.html) and NCBI (http://www.ncbi.nlm.nih.gov).

[c]Theoretical molecular mass (kDa) and pI were calculated using the ExPASy compute pI/Mw tool (http://ca.expasy.org/tools/pi_tool.html).

[d]Probability Based Mowse Score is −10×Log(P), where P is the probability that the observed match is a random event. Only peptides with individual scores greater than 30, which is indicative of significant homology, are presented in this table.

[e]Percent of protein sequence covered by matched peptides.

Thirteen proteins that were expressed only in the wild-type strain were identified (Table 2). Seven protein spots did not yield informative data owing to failed sequencing or lack of a significant match in the database. Of the thirteen proteins that we sequenced, six proteins matched an EST in the *F. verticillioides* Gene Index, whereas seven other proteins shared highest similarity to *F. verticillioides* auto-annotated genes. We also performed BLASTP analysis against the NCBI database to obtain the putative function of these genes in *F. verticillioides*. Ironically, when the protein sequences were searched against the NCBI nr database, no protein was suggested to play a direct role in fungal secondary metabolism. However, we did observe a high number of sequences that are associated with protein metabolism. A group of proteins that could be the target of further investigation is the peptidases. Peptidases are involved in a variety of cellular functions, including protein turnover, development, and gene signaling [29].

Table 3 describes proteins that are expressed only in the FT536 strain. Four protein spots did not yield informative data owing to failed sequencing or lack of a significant match in the database. Of the sixteen proteins that we obtained, sequences from nine peptides matched ESTs in the *F. verticillioides* Gene Index. Seven proteins shared highest similarity to *F. verticillioides* auto-annotated genes. Again, we performed BLASTP analysis against the NCBI nr database to obtain the putative function of these genes in *F. verticillioides*. Four of the identified proteins play a role in fungal secondary metabolism. Another significant category of proteins listed in Table 3 is proteins involved in stress response, such as heat-shock protein and superoxide dismutase.

Sequencing results of eight proteins that are differentially expressed, four from the wild type and four from FT536, are provided in Table 4. Imaging analysis showed that these proteins are expressed at greater than 5× level in either wild type or FT536 (Fig. 2). Only three protein spots from the wild-type strain gave sequence information, and those sequenced all matched significantly to the EST in the *F. verticillioides* Gene Index. All four FT536 proteins were sequenced. One sequence matched the EST in the *F. verticillioides* Gene Index whereas three others shared highest similarity to *F. verticillioides* auto-annotated genes.

Table 3. Proteins uniquely expressed in FT536 protein samples.

Spot no.	MS method	*F. verticillioides* locus match[a]	Putative function[b]	Mol. mass[c]	pI[c]	Mascot score[d]	Sequence coverage[e]
F1	Q-TOF	FVEG_12358.3	Putative laccase	29.359	6.62	40	10
F2	MALDI	FVEG_10369.3	Elongation factor 3	116.738	5.58	80	8
F3	MALDI	FVEG_12776.3	Pyruvate kinase	59.784	5.81	146	38
F4	MALDI	FVEG_10153.3	Phosphoglycerate kinase	44.882	5.91	180	39
F5	Q-TOF	No match	–	–	–	–	–
F6	Q-TOF	No match	–	–	–	–	–
F7	Q-TOF	FVEG_00289.3	Reductase or Zn-dependent oxidoreductase	33.824	7.85	48	2
F8	MALDI	FVEG_01253.3	Nitrogen metabolite repression-(nmr)-protein	94.215	4.50	85	14
F9	MALDI	FVEG_09479.3	Minor allergen Alt a 7	36.328	6.10	108	37
F10	Q-TOF	FVEG_07201.3	Oxidoreductase	31.746	6.52	459	38
F11	Q-TOF	FVEG_10626.3	Methyltransferase	31.290	6.44	173	18
F12	Q-TOF	No sequence	–	–	–	–	–
F13	Q-TOF	FVEG_05159.3	60S acidic ribosomal protein P0	24.462	5.14	41	9
F14	Q-TOF	No sequence	–	–	–	–	–
F15	Q-TOF	FVEG_07470.3	Heat-shock protein 90	79.818	4.93	69	4
F16	Q-TOF	FVEG_00186.3	Glutathione-*S*-transferase	28.371	10.76	89	8
F17	Q-TOF	No match	–	–	–	–	–
F18	Q-TOF	FVEG_11192.3	Superoxide dismutase, mitochondrial precursor	22.474	9.06	43	8
F19	Q-TOF	FVEG_11192.3	Superoxide dismutase, mitochondrial precursor	22.474	9.06	43	8
F20	Q-TOF	No match	–	–	–	–	–

[a]Gene locus according to the *F. verticillioides* genome database at the Broad Institute of MIT and Harvard (http://www.broad.mit.edu/annotation/genome/fusarium_verticillioides/Home.html).

[a]Descriptions of putative function were obtained from the *F. verticillioides* genome database at the Broad Institute of MIT and Harvard (http://www.broad.mit.edu/annotation/genome/fusarium_verticillioides/Home.html) and NCBI (http:/www.ncbi.nlm.nih.gov).

[f]Theoretical molecular mass (kDa) and pI were calculated using the ExPASy compute pI/Mw tool (http://ca.expasy.org/tools/pi_tool.html).

[d]Probability Based Mowse Score is $-10\times\text{Log}(P)$, where P is the probability that the observed match is a random event. Only peptides with individual scores greater than 30, which is indicative of significant homology, are presented in this table.

[e]Percent of protein sequence covered by matched peptides.

Table 4. Proteins differentially expressed in wild-type and FT536 protein samples.

Spot no.	MS method	*F. verticillioides* locus match[a]	Putative function[b]	Mol. mass[c]	pI[c]	Mascot score[d]	Sequence coverage[e]
W1915	Q-TOF	FVEG_04402.3	Serine carboxypeptidase	32.073	6.32	142	26
W3110	Q-TOF	FVEG_01921.3	Aminopeptidase	28.010	6.01	302	54
W5111	Q-TOF	FVEG_12529.3	*O*-Acetylhomoserine	28.430	6.21	160	21
W8907	Q-TOF	No match	–	–	–	–	–
F148	Q-TOF	FVEG_01986.3	Similar to peroxisomal membrane protein	13.722	7.93	55	16
F2719	Q-TOF	FVEG_01481.3	Enolase	47.538	5.01	76	27
F5611	MALDI	FVEG_00882.3	*S*-Adenosylmethionine synthetase 2	44.271	5.71	93	22
F5906	MALDI	FVEG_02780.3	Transketolase 1	74.824	5.53	86	15

[a]Gene locus according to the *F. verticillioides* genome database at the Broad Institute of MIT and Harvard (http://www.broad.mit.edu/annotation/genome/fusarium_verticillioides/Home.html).

[b]Descriptions of putative function were obtained from the *F. verticillioides* genome database at the Broad Institute of MIT and Harvard (http://www.broad.mit.edu/annotation/genome/fusarium_verticillioides/Home.html) and NCBI (http:/www.ncbi.nlm.nih.gov).

[c]Theoretical molecular mass (kDa) and pI were calculated using the ExPASy compute pI/Mw tool (http://ca.expasy.org/tools/pi_tool.html).

[d]Probability Based Mowse Score is $-10\times\text{Log}(P)$, where P is the probability that the observed match is a random event. Only peptides with individual scores greater than 30, which is indicative of significant homology, are presented in this table.

[e]Percent of protein sequence covered by matched peptides.

Table 5. Transcription levels of genes corresponding to selected protein spots.[a,c]

Protein spots	Gene expression in wild type[b]	Gene expression in FT536[b]
W3	1.0 (0.83–1.2)	0.5 (0.41–0.6)
W4	1.0 (0.9–1.1)	0.23 (0.18–0.29)
W10	1.0 (0.37–2.71)	1.83 (1.71–1.95)
W19	1.0 (0.63–1.58)	1.46 (1.16–1.84)
W20	1.0 (0.23–4.43)	2.26 (1.58–3.25)
F1	1.0 (0.62–1.61)	18.9 (7.57–47)
F8	1.0 (0.78–1.28)	0.58 (0.56–0.62)
F10	1.0 (0.64–1.57)	133.44 (116.97–152.22)
F11	1.0 (0.62–1.61)	7082.29 (5752.61–8719.32)
F15	1.0 (0.72–1.39)	0.8 (0.78–0.83)
F19	1.0 (0.6–1.68)	2.08 (2.03–2.14)
W1915	1.0 (0.69–1.45)	0.08 (0.07–0.09)
W3110	1.0 (0.71–1.41)	0.27 (0.16–0.47)
W5111	1.0 (0.53–1.88)	1.12 (0.81–1.54)
F148	1.0 (0.83–1.2)	0.66 (0.63–0.68)
F2719	1.0 (0.83–1.2)	1.83 (1.5–2.23)
F5611	1.0 (0.52–1.93)	0.48 (0.35–0.68)
F5906	1.0 (0.92–1.09)	0.43 (0.37–0.5)

[a]Total RNA samples were prepared from fungal strains grown on BSAL medium [26]. Quantitative real-time (QRT)-PCR analysis of gene expression was performed with SYBR-Green as the fluorescent reporter. The expression of each gene was normalized to endogenous β-tubulin (*TUB2*) (GenBank U27303) gene expression.

[b]The gene expression was calibrated using $2^{-\Delta\Delta Ct}$ [19]. Data represent the fold differences, where gene expression in the wild type is standardized to 1.0. The range of expression was calibrated using $2^{-\Delta\Delta Ct-s}$-$2^{-\Delta\Delta CT+s}$ where s is the standard deviation of ΔCt value. Ct=Threshold Cycle.

[c]Each value is the mean of three technical replicates from one biological experiment. A biological replication was performed with no statistically different results.

Transcriptional Profile of Genes that Correspond to the Differentially Expressed Proteins

After the identification of the differentially expressed proteins, we tested the transcription level of each corresponding gene with qRT-PCR. *F. verticillioides* EST sequences corresponding to the 18 identified proteins were selected for gene expression study. Of the five proteins identified in the wild-type 2D-PAGE, only W3 and W4 showed higher transcription levels in the wild-type strain (Table 5). Three other sequences (W10, W19, and W20) showed contradictory transcriptional and translational expression patterns. Of the six proteins identified from the FT536 2D-PAGE, four sequences (F1, F10, F11, and F19) showed higher mRNA and protein expressions in FT536 (Table 5). Notably, F10 and F11 showed greater than 100-fold transcriptional upregulation in the mutant. Genes encoding F8 and F15, on the other hand, showed higher gene expression in the wild type. When transcriptional patterns of genes corresponding to the differentially expressed proteins (Table 4) were analyzed, we observed that no significant transcriptional-translational correlation is present (Table 5). This study demonstrates to us that the level of protein expression in a fungal cell is not directly dependent upon the gene expression level.

Discussion

Proteomic studies have been performed on a variety of microbes under different environmental and nutritional conditions as well as genetic backgrounds [10, 17, 30, 33]. These studies have revealed valuable molecular genetic data that went unnoticed or overlooked previously. The premise of this investigation was that by identifying proteins

that are differentially translated in *F. verticillioides* wild type and FT536, we would gain additional molecular genetic information associated with FB_1 biosynthesis and fungal development. In our study, we were able to obtain corresponding gene sequences of 42 proteins that were differentially expressed (>5 folds) in the wild type and FT536. Differential expressions of these proteins were verified by two biological replications and three independent technical replications of 2D-PAGE before performing protein spot isolation and subsequent MS analyses. Of the 42 proteins that were sequenced, 16 proteins were unique to the wild type, 17 were unique to FT536, 4 were upregulated in the wild type, and 4 were upregulated in FT536. Based on the sequence information retrieved, we categorized the proteins into putative functional groups, *e.g.*, protein metabolism, secondary metabolism, stress response, and signal transduction. Contrary to our anticipation, we did not observe a high number of proteins associated with secondary metabolism in the wild type. This result is noteworthy, since it is in contrast to the transcriptional data obtained in SSH and microarray studies, where a number of *FUM* genes were identified in the wild-type samples [26, 35]. A relatively high number of protein sequences that are associated with fungal stress responsiveness were identified in the FT536 strain, and this observation is in agreement with the previous SSH and microarray studies [26, 35] where a high number of stress responsive genes were identified in the mutant genetic background. Our results reaffirm the importance of a functional *FCC1* for *F. verticillioides* to cope with ambient environmental stress factors.

In the wild-type strain, proteins with a functional role in FB_1 biosynthesis were not identified, but rather, a relatively high number of proteins known to be associated with protein metabolism. Among the proteins identified in the wild-type strain proteome were protein peptidases (W6, W1915, W3110). Protein peptidases are known to perform a wide variety of cellular functions, such as protein turnover, enzyme modification, and regulation of gene expression [29]. Moreover, recent published reports suggest that post-translational modification of enzymes associated with secondary metabolite biosynthesis may play an important role than previously anticipated [7, 18, 39]. For example, *Aspergillus fumigatus* protein Ayg1, which contains a serine protease-type hydrolytic motif, is involved in formation of a pentaketide 1,3,6,8-tetrahydroxynaphthalene (T4HN), which is a key precursor of 1,8-dihydroxynaphthalenemelanin, an important virulence factor in pathogenic fungi [7]. We anticipate that protein peptidases identified in this study may possess proteolytic activities on putative enzymes that influence FB_1 biosynthesis. In addition, proteolytic modification of enzyme precursors may help activate particular functions or enable proper protein trafficking, which will ultimately contribute to regulating secondary metabolism and cellular development in *F. verticillioides*.

Our proteomic analysis also revealed several interesting genes upregulated in FT536 that are candidates for further investigation. One example is the superoxide dismutase identified in Table 3 (F18, F19). Reactive oxygen species are generated under stress conditions, and in many organisms, superoxide dismutase plays an indispensable role in protecting organisms from the reactive oxygen species. The importance of this enzyme in biological systems was clearly demonstrated in a study by Li *et al.* [16], in which a null mutation of superoxide dismutase in mice led to lethality. It is also reported that Mn^{2+}-containing superoxide dismutase is involved in the protection of *Candida albicans* during the stationary growth stage when a dramatic burst of reactive oxygen species is known to occur [14]. Hence, the upregulation of the putative superoxide dismutase in FT536 suggests that the mutant is actively responding to internal or external stress. Moreover, reactive oxygen species are known to affect sexual development in *Aspergillus nidulans* [15], and therefore, we can hypothesize that the superoxide dismutase is negatively impacting conidiation in FT536 [35]. Another interesting protein identified in FT536 2D-PAGE is glutathione S-transferase (F16). Although glutathione S-transferase is widely used as an enzyme to purify protein of interest, its cellular function is to act as a detoxification enzyme. All eukaryotes possess multiple glutathione S-transferase isozymes, which underscores the importance of this enzyme in the cellular detoxification process [9]. In *A. nidulans*, glutathione S-transferase contributes to resistance against heavy metal and xenobiotic stresses [6]. Perhaps similar to superoxide dismutase, the role of glutathione S-transferase is to relieve endogenous (or exogenous) stress in FT536. On the other hand, it has been shown in parsley (*Petroselinum crispum*) that glutathione S-transferase is involved in flavonoid production gene signaling [20]. Further functional characterization is necessary to verify the role of glutathione S-transferase in *F. verticillioides* secondary metabolism.

Notably, we also identified a protein (F8) that is highly similar to the *F. fujikuroi* nitrogen metabolite repression-responsible protein (Table 3). Nitrogen metabolite repression is a global regulatory mechanism for the selection of preferred nitrogen source. Significantly, nitrogen metabolite repression and secondary metabolism in filamentous fungi have been closely linked, including fumonisin biosynthesis [2, 34, 37]. Although our understanding of how nitrogen metabolite repression impacts fungal secondary metabolism gene signaling pathways in fungi is far from complete, it is reasonable to anticipate that elevated expression of the nitrogen metabolite repression-responsible protein is having a negative impact on FB_1 biosynthesis in FT536. Additionally, proteins known to play a role in secondary metabolism, oxidoreductase (F10) and methyltransferase A (F11), were

identified in the FT536 proteome. Oxidoreductase and methyltransferase are similar to enzymes involved in aflatoxin biosynthesis in *Aspergillus parasiticus* and *A. flavus* [3, 12]. Differential expression of these enzymes is not totally surprising since *F. verticillioides* produces a variety of secondary metabolites other than FB_1, and it can be anticipated that the oxidoreductase and methyltransferase A identified in the FT536 proteome are involved in synthesizing other secondary metabolites, perhaps pigments or other mycotoxins. Comparative genomic analysis of secondary metabolite gene clusters in *Fusarium* species is in progress to help us associate these enzymes to certain secondary metabolism pathways.

We followed up *in silico* analysis with qRT-PCR to investigate the transcriptional pattern of genes that encode the identified proteins. Correlation between transcription and translation is known to be less than 50% [13], and we observed a similar trend when we analyzed the transcription levels of the identified proteins. Among the 18 proteins selected for the gene expression study, only 8 gene/protein samples (44.4%) showed consistent upregulation in both mRNA and protein expressions. We grouped the proteins into three categories according to gene expression-protein expression pattern: positive correlation, negative correlation, and no significant correlation. Although we anticipate that gene-protein pairs that show positive correlation are primary candidates for functional analysis, we cannot rule out the possibility that proteins that fall into other categories are associated with *F. verticillioides* secondary metabolism. In other work, we have been generating knockout mutants of *F. verticillioides* genes discovered *via* microarray analysis [26], but a relatively high number of these mutants show no deviation from the phenotype of wild-type progenitor [Choi and Shim, unpublished data]. Currently, we are in the process of characterizing the functional role of genes identified in this study to assess their association with FB_1 biosynthesis.

To summarize, we used a proteomic approach to elucidate additional genetic information that may have potential roles in fumonisin biosynthesis in *F. verticillioides*. *In silico* analysis suggests that several proteins identified in the 2D-PAGE may play a role in secondary metabolism and stress responsiveness. Moreover, we followed up the proteomic analysis with qRT-PCR analysis to perform comparative analyses of transcriptional and translational expressions in *F. verticillioides*. To our knowledge, this is the first demonstration of utilizing a proteomic approach to isolate novel genes associated with secondary metabolism and development in *F. verticillioides*. Functional characterization of the identified genes/proteins is in progress to further validate the functional role of the identified genes in *F. verticillioides* secondary metabolism.

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