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RESEARCH ARTICLE

Alteration in the gene expression of *Glehnia littoralis* seedlings exposed to culture filtrate of *Penicillium citrinum* KACC43900

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Plant growth and gibberellins (GAs) biosynthesis are two separate but linked processes, involving many genes but fewer have been reported for their role in plant growth and development. Due to little information on the genes involved in such processes, the known plant growth promoting and GAs producing fungal endophyte *Penicillium citrinum* KACC43900 was used as potential tool to obtain a blueprint of the putative growth promoting and GAs synthesizing proteins. For proteomic analysis, the seedlings of *Glehnia littoralis* were treated with culture filtrate of *P. citrinum* KACC43900, which revealed significant differences between 2 dimensional gel electrophoresis profile of the crude protein extracts of the treated and control samples. Matrix-assisted laser desorption/ionization analysis of the 56 selected spots led to the identification of 41 proteins. A significant number (31.5%) of these highly expressed proteins were associated with plant growth regulation, including beta-expansin EXPB4, ent-kaur 16-ene synthase, gibberellin 3-oxidase, and cytochrome P450 family proteins. Proteins involved in regulating energy metabolism and intracellular redox conditions, such as glyceraldehyde-3-phosphate dehydrogenase and ribulose-1,5-bisphospahate carboxylase/oxygenase, were also expressed. It was concluded that culture filtrate of *P. citrinum* KACC43900 altered the gene expression pattern of host *G. littoralis*. Current study highlighted the importance of proteomics as a starting tool for any post-genomic research.

Keywords: *G. littoralis*; *Penicillium citrinum* KACC43900; proteomics; gibberellins; plant growth

Introduction

The plant growth is regulated by naturally occurring compounds that are effective in very minute concentrations, and known as phytohormones. Several diverse groups of plant soil microbes, including fungi, have been reported to promote plant growth by producing a wide array of compounds related to plant growth and immunity (El-Tarabily et al. 2009; Richardson et al. 2009; Waqas et al. 2014). Beside several approaches used to understand the complex process of growth and development elicit by plant growth promoting organisms (Wu et al. 2009; Doty 2011; Hussain & Hasnain 2011; Gamalero & Glick 2011), several aspects are still elusive. Gibberellins (GAs) constitute an important group of plant growth regulators, produced by growth promoting endophytic fungi, and involve a large number of genes with fewer been reported for their respective roles (Depuydt & Hardtke 2011; Khan et al. 2014).

Being major players in food and pharmaceutical industry, intense interest surrounds the members of kingdom fungi, such as filamentous fungi and yeasts. The enormous biodiversity within the mycota has resulted in their application as model organisms for the production of fuels, chemicals, food ingredients, pharmaceuticals, and enzymes. Equally important is the central role that fungi occupy as model systems for basic research. The relevance of fungi in food and feed spoilage (primarily through mycotoxin production) and their pathogenicity has also elevated the importance of fungal research (Jewett et al. 2006).

Penicillium citrinum KACC43900, a GAs producing endophytic fungus, was isolated from the roots of sand dune plant *Ixeris repens* (Khan et al. 2008). The secondary metabolites (GAs) are not genetically encoded and are substrates, intermediates, or product of metabolism. The metabolic pathways are, however, catalyzed by several enzymes (functional proteins) encoded by specific genes. Researchers use genomic or proteomic or both approaches to verify the production of the target metabolites. Proteomics is the study of the proteome, which is the total protein complement of a genome. Unlike the genome, which is essentially similar in all somatic cells of an organism, the proteome is a dynamic entity that is different in different cell types, and changes with the physiological phenotypic expression of the genomic information. An important advantage of global protein expression profiling as compared to individual

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gene regulation is the ability to monitor changes in several functional groups simultaneously (Jiang et al. 2008).

We selected *Glehnia littoralis* in order to analyze alteration in its gene expression, when treated with culture filtrate of *P. citrinum* KACC43900. *G. littoralis* is a perennial herb, belong to family Appiaceae, and grow wild on the sandy shores of China, Japan, and Korea. The plant is edible and also known for its therapeutic activity. *Glehnia* root has been listed in the Japanese and Chinese pharmacopoeias, and the plant has been used as diaphoretic, antipyretic, and analgesic in the traditional medicine of these countries. Aerial parts are used as salad and as aromatic vegetable (Miyazawa et al. 2001). Hatta et al. (1967a, 1967b) reported that the germination rates, seed weight, root yield, extract yield, and chemical components of the cultivated plants of *G. littoralis* varied depending upon the place of growth of the plants. The variation may be caused by genetic, environmental, and physiological factors. The subterranean parts of *G. littoralis* are affected by factors such as the duration of growth, moisture content of soil, atmospheric temperature, and concentration of macronutrients.

Proteomic techniques such as two-dimensional gel electrophoresis (2D-gel electrophoresis) and mass spectrometry (MS) can be used to characterize and quantify molecular responses of an organism to environmental stimuli. In particular, 2D-gel electrophoresis is a method that can be used to accurately quantify protein expression differences under various conditions (Westermeier 2006). The proteomic approach helps to understand the pattern of proteins, with altered expression, which should be focused on for a better understanding of the possible mechanisms involved in the protein changes brought about under the influence of phytohormones. Due to the difficulty associated with genomic approach, in order to obtain a global image of the genes linking plant growth and GAs (Vanhaeren et al. 2014), we adopted proteomic approach to obtain a blueprint of the putative growth promoting proteins. For this purpose growth promoting and GAs producing *P. citrinum* KACC43900 was used to modulate the expression of proteins involved in plant growth and development.

Materials and methods

Collection of G. littoralis seeds

Mature seeds of sand dune plant *G. littoralis* were collected from the coastal dunes of Pohang, Korea. Seeds were sealed in separate paper bags, labeled, and transported to Microbial Genomics Laboratory, Kyungpook National University, where seeds were dried at ambient temperature and stored at 4 °C till further processing.

Growth medium and culture conditions for P. citrinum

For secondary metabolites production, *P. citrinum* KACC43900 was grown on Czapek broth medium (1% glucose, 1% peptone, 0.05% KCl, 0.05% $MgSO_4 \cdot 7H_2O$, and 0.001% $FeSO_4 \cdot 7H_2O$, pH 7.3±0.2) by incubating on a shaking incubator set at 30 °C with 120 rpm for 7 days.

Seed dormancy and germination

The seeds of *G. littoralis* were surface sterilized following an established protocol (Mineo 1990). Various seed dormancy breaking methods including cold treatment, hot water treatment, and sulfuric acid treatment were applied. The seeds were checked daily for the emergence of radicle.

Treatment of G. littoralis seedlings with fungal culture filtrate (CF)

To assess the growth promoting capacity of the secondary metabolites produced by *P. citrinum* KACC43900, seedlings of *G. littoralis* were assayed with fungal CF (Khan et al. 2008). Seedlings of uniform size were selected and sown in perlite–vermiculite (1:1) mixture and kept in the greenhouse to grow. Hoagland nutrient solution (Hoagland & Arnon 1950) was given to these seedlings on sixth day of sowing. An amount of 20 µL fungal CF was applied at apical-meristem of the shoot, and incubated for 7 days to observe its effect on shoot elongation. After 7 days of fungal CF application, the seedlings were harvested, shoot and plant length parameters were recorded, and compared with control.

Protein extraction, gel electrophoresis, and spot analysis

G. littoralis seedlings were freezed at −80 °C and later grounded to fine powder in liquid nitrogen. Proteins were extracted using potassium phosphate buffer solubilization and trichloroacetic acid precipitation (Tsugita & Kamo 1999), and were quantified following the Bradford (1976) method, with the Coomassie protein assay reagent kit (Pierce, USA) keeping bovine serum albumin as a standard. Protein extract of both samples were separated by 2-DE, following an established protocol (Shen et al. 2002). The gels were stained with 0.1% Coomassie Brilliant Blue R-250 as indicated by Scheler et al. (1998). The stained gels were scanned using a UMAX Power Look 2100XL scanner (Maxium Tech, Taipei) at 600 dpi in transparency mode to obtain a gray scale image at the following parameters: saliency 2, partial threshold 4, and minimum area 50. The spots were named with ordinal numbers. The spot volumes (%) of the two samples (control and fungal CF treatment) were compared using two samples Student's *t*-test, and only significantly changed protein spots ($p < 0.05$) were subjected to protein identification. For the selected spots the following parameters were assessed: isoelectric point (pI), molecular mass, mean values of protein amount, standard deviation as a measure of dispersion, and coefficient of variance.

Protein identification

For identification, the selected protein spots were excised and in-gel digested as previously described (Shevchenko et al. 1996), using modified porcine trypsin method. Gel pieces were washed with 50% acetonitrile to remove sodium dodecyl sulfate, salts, and stain. The washed gel pieces were dried to remove solvent and then rehydrated with 10 µL of trypsin solution (10 ng μL^{-1}) and incubated overnight at 37 °C. The proteolytic reaction was ended by addition of 5 µL of 0.5% trifluoroacetic acid. Tryptic peptides were recovered by combining the aqueous phase from several extractions of gel pieces with 50% aqueous acetonitrile. The peptide mixture was concentrated and desalted using C_{18}ZipTips (Millipore), and peptides eluted in 1–5 µL of acetonitrile. An aliquot of this solution was mixed with an equal volume of a saturated solution of α-cyano-4-hydroxy-cinnamic acid in 50% aqueous acetonitrile, and 1 µL of mixture was spotted on the sample plate of mass spectrometer. The samples were analyzed by matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) MS. For each sample, mass calibration was done using trypsin auto-digestion peaks *m/z* (842.510, 2211.1046) as internal standards. Proteins were identified by submitting the peptide mass finger-printing data to the search software ProFound of The Rockefeller University (http://prowl.rockefeller.edu/prowl-cgi/profound.exe).

Results and discussion

Breaking seed dormancy of G. littoralis

Sand dune plants usually exhibit seed dormancy as a tool of survival in the harsh environments (Kadis & Georghiou 2010), and germinate after long-term water soaking treatment. *G. littoralis* is a reported seed dormant plant (Yu-ling 2008), and cold treatment of *G. littoralis* seeds, wrapped in moistened muslin cloth incubated at 4 °C for two weeks in dark proved to be the most efficient method of breaking seed dormancy, resulting in the emergence of radicles in three months after such treatment. Substantial improvement in the rate of emergence of radicles in *G. littoralis* seeds after cold treatment has been previously reported (Liu et al. 2004)

Effect of fungal CF on seedling growth of G. littoralis

G. littoralis seedlings of uniform size were shifted to perlite–vermiculite (1:1) mixture and allowed to grow in greenhouse conditions. The seedlings attained two-leaf stage at 25th day after the application of nutrient solution. We observed very little differences between the shoot lengths of fungal CF treated and control seedlings after one week of incubation in the greenhouse (Figure 1). Sand dune plants show slow growth rate, especially their shoots as compared to roots (Kunkel 1984), and it could possibly be the reason for not observing noticeable shoot length differences in the treated and control samples.

Comparison of 2DE protein profiles and spot analysis

About 100 reproducible protein spots with the mass range of 10–113 kDa were detected on gel images of fungal CF treated and control seedlings of *G. littoralis*. We selected 56 protein spots on fungal CF treated sample gel (Figure 2a) on the basis of their high (41 spots) or unique expression (15 spots), as compared to control (Figure 2b). For the highly expressed proteins, the difference in the intensity between fungal CF treated and control gels ranged from 1.49 to 37.26 folds (see Table 1). The uniquely expressed protein spots appeared exclusively on fungal CF treated sample gel as mentioned (see Table 2).

MALDI-TOF analysis and database search

Using the ProFound search database, 52/56 selected spots were identified from the MALDI-TOF MS data. The identified proteins are listed in Tables 1 and 2, and numbered on the virtual 2 dimensional gel electrophoresis (2DE) reference map (Figure 2). MALDI-TOF MS analysis (Tables 1 and 2) allowed significant homology matches of 52 out of 56 spots (92.8% success rate). Earlier, 82.3% success rates for protein identification using MALDI-TOF MS has been reported (Ferreira et al. 2006). This technique thus made it possible to achieve the best homology match for protein identification. Several peptide sequences for the same protein enhanced the information and allowed higher accuracy of matches as compared to the use of single sequence data.

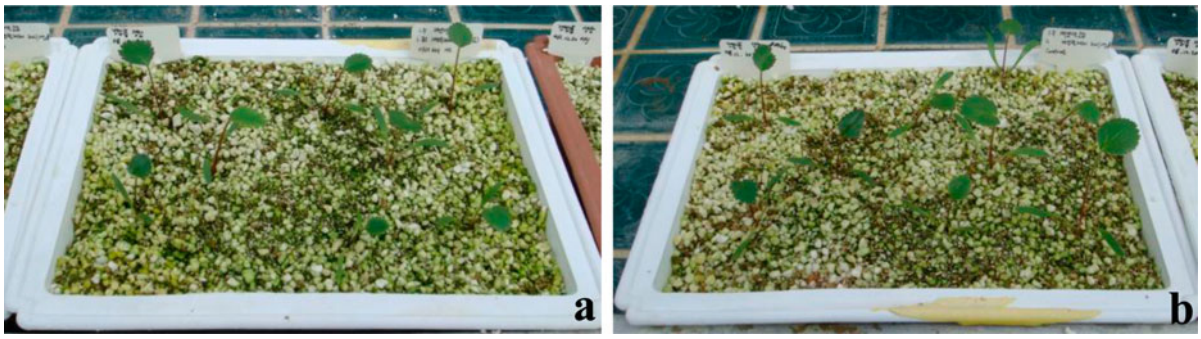

Figure 1. Forty days old seedlings of *G. littoralis* treated with culture filtrate of fungal endophyte *P. citrinum* KACC43900 and (b) control treatment.

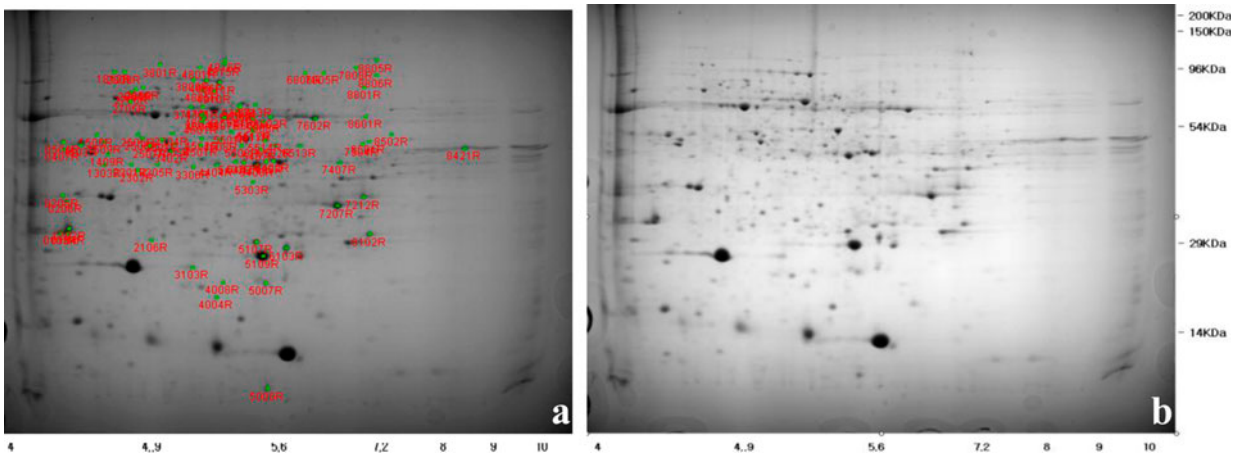

Figure 2. Comparison of 2DE profiles of crude protein extracts from fungal CF treated (a) and control (b) seedlings of *G. littoralis*. Image analysis showed difference in the intensity of 100 protein spots with mass range between 10 and 113 kDa. Fifty six spots with statistically significant difference were analyzed with MALDI-TOF MS for protein identification.

Functional categories of the identified proteins and their role

Out of 56 selected spots, 52 spots led to the identification of 41 proteins. These proteins were classified into six categories on the basis of their role in different processes, including growth associated proteins (GAP), photosynthesis and associated proteins, protein synthesis and associated proteins, transport associated proteins (TP), energy metabolism associated proteins, and miscellaneous proteins (MP). Most of the identified proteins (31.5%) were related to plant growth and development, and increased metabolic activity (Figure 3). Glyceraldehyde-3-phosphate, ribulose-1,6-bisphosphate carboxylase, phosphoglycerate kinase, ornithine decarboxylase (ODC), nicotinamide adenine dinucleotide phosphate (NADPH)-quinone oxidase-reductase, uridine diphosphate (UDP)-glucose: glucosyltransferase, and glutamate-1-semialdehyde-2,1-aminomutase were highly expressed proteins found in fungal CF treated samples. These proteins are associated with metabolism, and indicate an increase in plant growth. A significant increase (37.26 fold) was observed in UDP-glucose: glucosyltransferase protein, which is known to control plant development by opposing glucuronidase activity, thereby modulating the duration of the cell cycle (Woo et al. 2007). Glyceraldehyde 3-phosphate dehydrogenase (GADPH) has been identified as multifunctional protein, and is designated as house-keeping enzyme. Functions attributed to this enzyme are gene transcription, DNA replication, nuclear ribonucleic acid (RNA) export, endocytosis, microtubule bundling, phosphotransferase, oncogenesis (Sirover 2005; Baek et al. 2008), and the key processes involved in growth and development. A highly expressed protein, ODC and uniquely expressed beta-expansin, EXPB4 were other important GAP implicated separately in the two growth process, the cell proliferation, and cell expansion. ODC is associated with plant cell multiplication (Cohen et al. 1982) and polyamine biosynthesis (Alcazar et al. 2010), while the beta-expansin EXPB4 is known to be the key regulators of cell expansion during growth (Lin et al. 2005), by modifying the cell wall (Jamet et al. 2008). Previous studies reported an increased activity and expression of expansions during GAs induced elongation in deep water rice (Choe & Cosgrove 2004), indicating the presence of GAs in the fungal CF. Among the uniquely expressed proteins in the seedlings exposed to fungal CF, was glycine rich protein, implicated in cell elongation in *Arabidopsis* (Mangeon et al. 2009). Other growth related proteins included the shoot elongation proteins, which either over expressed (e.g. gibberellin 3-oxidase and cytochrome P450 family protein) or uniquely expressed (e.g. ent-kaur 16-ene synthase) in the treated samples. More than 20-fold increase was recorded in the expression level of gibberellin 3-oxidase in treated samples (Table 1), which is responsible for the synthesis of bioactive forms of GAs during vegetative plant growth (Mitchum et al. 2006). Cytochrome P450 family protein are partners in a broad range of biosynthetic reactions in plants, resulting in conjugated lipids, defensive compounds, and plant growth regulators (Werck-Reichhart et al. 2000). The uniquely expressed, ent-kaur 16-ene synthase affect plant growth by catalyzing the synthesis of ent-kaur 16-ene, a gibberellin biosynthesis precursor, from ent-copalyl diphosphate (Xu et al. 2007), thus indicating the presence of GAs inducing secondary metabolites in the fungal CF. Citrate synthase like protein was expressed uniquely in the treated plants, a reportedly GAP in *Arabidopsis thaliana* (Koyama et al. 2000). Consistent with our results, exogenous application of GAs resulted in increased expression of this enzyme in young seedlings of castor bean (Gonzalez & Delsol 1981; Kagawa & Gonzalez 1981) and *Ricinus* (Gonzalez & Delsol 1981; Kagawa & Gonzalez 1981).

Application of fungal CF to the *G. littoralis* seedlings also enhanced the expression level of proteins involved in signaling of plant hormones. Among them was seventeen kilodalton protein (SKP)1/Arabidopsis SKP1 (ASK) interacting protein, known to be involved in auxins, jasmonic acid, and GAs signaling (Santner et al. 2009). Likewise, the leucine rich repeat, extracellular domains of

Table 1. Highly expressed proteins identified from *G. littoralis* seedlings treated with culture filtrate of *P. citrinum*.

Spot no.[a]	Mol.wt./pI[b]	Identification	Category	Plant[c]	Accession no.[d]	Fold[e]
103	32.31/4.27	Chlorophyll a b binding protein	PAP	*Apium graveolens*	P92919	2.70
205	39.20/4.28	Ribosomal protein S3	PSAP	*Halimeda simulans*	AAV83771	1.94
1812	99.42/4.61	Hypothetical protein	UF	–	–	3.82
2305	45.55/4.86	Glyceraldehyde-3-phosphate dehydrogenase A subunit	GAP	*Arabidopsis thaliana*	NP_566796	5.35
2407	50.14/4.87	Phosphoglycerate kinase	GAP	*Arabidopsis thaliana*	NP_176015	3.97
2505	54.24/4.74	Ribulose-1,5-bisphosphate carboxylase/ oxygenase	PAP	*Arabidopsis thaliana*	NP_051067	–
2706	81.85/4.71	Sex determination transformer protein 2 precursor	MP	*Arabidopsis thaliana*	AAD29784	1.89
2804	84.79/4.73	Ornithine decarboxylase	GAP	*Arabidopsis thaliana*	NP_001234616	2.76
3306	45.46/5.06	Glyceraldehyde-3-phosphate dehydrogenase A subunit	GAP	*Arabidopsis thaliana*	NP_566796	2.55
3402	49.60/4.94	Maturase K	PSAP	*Medicago trancatula*	XP_003621693	1.83
3505	51.53/4.95	Heat shock protein binding protein	MP	*Arabidopsis thaliana*	NP_849392	2.61
3711	71.75/5.06	ATP-synthase CF1 alpha subunit	EAP	*Arabidopsis thaliana*	NP_051044	3.03
3808	94.11/5.06	SKP1/ASK-interacting protein 16	GAP	*Arabidopsis lyrata*	XP_002892320	3.11
4004	20.51/5.19	Ribulose-1,5-bisphosphate carboxylase/ oxygenase	PAP	*Arabidopsis thaliana*	NP_051067	3.13
4008	22.83/5.23	ATKT1 putative K ion transporter	TAP	*Arabidopsis thaliana*	AAF14830	2.14
4404	46.18/5.19	GBF2, G-box binding factor 2	PSAP	*Arabidopsis thaliana*	NP_192021	4.33
4502	53.40/5.11	Putative retroelement polyprotein	MP	*Arabidopsis thaliana*	AAG10817	2.01
4601	62.50/5.11	Leucine rich repeat family protein	GAP	*Arabidopsis thaliana*	NP_190892	2.79
4701	72.82/5.12	ATP-synthase CF1 alpha subunit	EAP	*Arabidopsis thaliana*	NP_051044	2.95
4801	105.76/5.10	ATP dependent CLPC heat shock protein	MP	*Arabidopsis thaliana*	NP_568746	2.05
4815	109.45/5.22	Phytochrome defective E	EAP	*Arabidopsis thaliana*	NP_193547	2.68
4816	113.64/5.23	NADPH-quinone oxidase-reductase subunit	EAP	*Malva viscus arboreus*	Q9MVL6	7.25
5002	10.37/5.36	UDP-glucose: glucosyltransferase	GAP	*Arabidopsis thaliana*	BAA34687	37.26
5107	30.08/5.42	Polypeptide of photosystem II	PAP	*Oryza sativa*	AAC98778	2.83
5109	27.58/5.46	Ribulose-1,5-bisphosphate carboxylase/ oxygenase	PAP	*Arabidopsis thaliana*	NP_051067	1.68
5303	41.93/5.39	Ribulose-1,5-bisphosphate carboxylase activase	PAP	*Nicotiana tabacum*	S25484	2.68
5408	46.23/5.42	Putative polynucleo adenylyltransferase	PSAP	*Arabidopsis thaliana*	NP_197758	2.54
5409	46.57/5.47	Putative polynucleo adenylyltransferase	PSAP	*Arabidopsis thaliana*	NP_197758	3.65
5508	51.36/5.33	Glutamate-1-semialdehyde-2, 1-aminomutase	GAP	*Arabidopsis thaliana*	NP_201162	1.93
5605	64.75/5.38	WRKY34 microspore specific promoter/ transcription factor	MP	*Arabidopsis thaliana*	NP_194374	2.06
5608	62.78/5.44	La domain containing protein	MP	*Arabidopsis thaliana*	NP_201411	3.08
5705	69.77/5.30	Acyl CoA thioesterase family protein		*Arabidopsis thaliana*	AEE81893	3.99
6103	28.79/5.59	K+ channel protein	TAP	*Arabidopsis thaliana*	AAA87294	1.81
6513	50.94/5.80	Glyceraldehyde-3-phosphate dehydrogenase B subunit	GAP	*Arabidopsis thaliana*	NP_174996	1.76
7212	38.37/6.78	Ribulose-1,5-bisphosphate carboxylase/ oxygenase	PAP	*Arabidopsis thaliana*	NP_051067	1.98
7407	46.40/6.40	Glyceraldehyde-3-phosphate dehydrogenase A subunit	GAP	*Arabidopsis thaliana*	NP_566796	1.62
7805	98.24/6.13	Cobalamine independent methionine synthase	PSAP	*Arabidopsis thaliana*	NP_197294	1.73
8102	31.30/6.89	Gibberellin 3-oxidase	GAP	*Solanum tuberosum*	ACN89835	20.01
8801	85.28/6.80	ATP dependent RNA helicase, putative	PSAP	*Arabidopsis thaliana*	AAG51496	1.49
8805	110.68/6.98	Cobalamine independent methionine synthase	PSAP	*Arabidopsis thaliana*	NP_197294	2.08
8806	95.24/6.99	Cytochrome P450 family protein	GAP	*Arabidopsis thaliana*	AEE77974	2.73

Note: The proteins were identified by submitting MALDI-TOF MS data to the search database ProFound.
GAP, growth associated proteins; PSAP, protein synthesis and associated proteins; PAP, photosynthesis associated proteins; EAP, energy metabolism associated proteins; TAP, transport associated proteins; MP, miscellaneous proteins; UFP, proteins with unknown function; ATP, adenosine triphosphate.
[a]Spot number on the gel image.
[b]The actual mass and pI of the protein determined on the gel.
[c]The plant of the highly matching proteins in the NCBI Protein database.
[d]The accession numbers of the highly matching proteins in the NCBI Protein database.
[e]Ratio of spot intensity on the gel of fungal CF treated samples and control samples.

Table 2. Uniquely expressed proteins identified from *G. littoralis* seedlings treated with culture filtrate of *P. citrinum*.

Spot no.[a]	Mol.wt./pI[b]	Identification	Category	Plant[c]	Accession no.[d]	Fold[e]
1202	32.94/4.34	Unknown protein	UF	*Arabidopsis thaliana*	NP_177271	8.48
2302	44.58/4.74	Hypothetical protein	UF	*Oryza sativa*	EEE67072	–
3506	57.02/4.96	Toprim domain containing protein	GAP	*Arabidopsis thaliana*	AEE31259	2.61
3801	108.04/4.88	Polyprotein	MP	*Oryza sativa*	AAT85010	–
5003	22.56/5.36	Potassium transporter, putative	TAP	*Arabidopsis thaliana*	AAF14830	–
5007	22.62/5.49	Hypothetical protein	UF	–	–	–
5407	50.26/5.40	VCS nucleotide binding protein	PSAP	*Arabidopsis thaliana*	AEE75331	–
5511	57.14/5.38	Glycine rich protein	GAP	*Arabidopsis thaliana*	CAA77883	–
5512	58.28/5.40	Endonuclease/exonuclease/phosphatase family protein	PSAP	*Arabidopsis thaliana*	NP_181918	–
5514	52.77/5.46	WRKY34 microspore specific promoter/transcription factor	MP	*Arabidopsis thaliana*	NP_194374	–
5713	73.49/5.40	Ent-kaur 16-ene synthase	GAP	*Arabidopsis thaliana*	AEE36246	–
6806	98.25/5.85	Cobalamine independent methionine synthase	PSAP	*Arabidopsis thaliana*	NP_197294	–
7504	51.23/6.77	Unnamed protein	UF	–	–	–
7808	103.23/6.66	Beta-expansin EXPB4	GAP	*Oryza sativa*	AAK55466	–
8502	54.01/7.22	Citrate synthase like protein	GAP	*Arabidopsis thaliana*	NP_850415	–

Note: The proteins were identified by submitting MALDI-TOF MS data to the search database ProFound.
GAP, growth associated proteins; PSAP, protein synthesis and associated proteins; PAP, photosynthesis associated proteins; EAP, energy metabolism associated proteins; TAP, transport-associated proteins; MP, miscellaneous proteins; UFP, proteins with unknown function.
[a]Spot number on the gel image.
[b]The actual mass and pI of the protein determined on the gel.
[c]The plant of the highly matching proteins in the NCBI Protein database.
[d]The accession numbers of the highly matching proteins in the NCBI Protein database.
[e]Ratio of spot intensity on the gel of fungal CF treated samples and control samples.

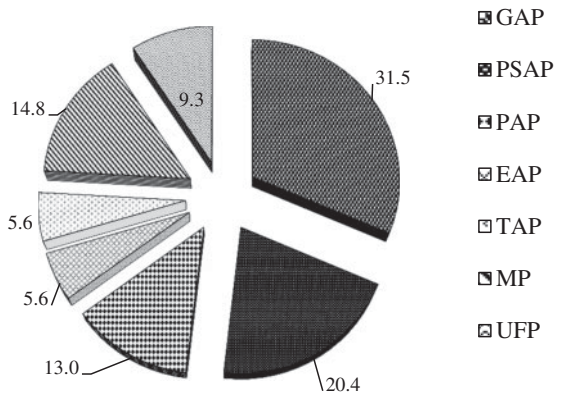

Figure 3. Proportion (%) of highly or uniquely expressed proteins groups (based upon their functions in plants) in fungal CF treated seedlings of *G. littoralis*. GAP, growth associated proteins; PSAP, protein synthesis and associated proteins; PAP, photosynthesis associated proteins; EAP, energy metabolism associated proteins; TAP, transport associated proteins; MP, miscellaneous proteins; UFP, proteins with unknown function.

receptor kinases, forming the largest family of receptors in plants, are implicated in brassinosteroids signaling and plant immunity (Jaillais et al. 2011; She et al. 2011). These results indicate enhanced hormonal crosstalk to induce growth in treated seedlings.

Proteins involved in photosynthesis or associated processes, represents important proportion of the proteins showing enhanced expression in the fungal CF treated seedlings. These include ribulose-1,5-bisphosphate carboxylase (Rubisco), a CO_2 fixation enzyme found in chloroplast of higher and lower photosynthesizing plants. An increased level of this protein induces rapid plant growth and promotes leaf growth and cholophyll contents. This enzyme also serve as storage protein that can be hydrolyzed during leaf senescence and utilized as a source of nitrogen for developing leaves and fruits in higher plants (Miller & Huffaker 1982).

Enhanced expression of Rubisco was coupled with the up-regulation of ribulose-1,5-bisphosphate carboxylase activase, which control photosynthesis by removing inhibitor from the catalytic site of Rubisco to regulate its activity (Portis 2003). Phosphoglycerate kinase is an important enzyme which functions in photosynthetic carbon metabolism, glycolysis, and gluconeogenesis. In green plants the enzyme has been shown to exist in the mitochondria, chloroplasts, and cytoplasm (Joyard et al. 2010).

Enzymes involved in bioenergetics were also up-regulated in seedlings supplied with fungal CF as compared to the control seedlings. NADPH-quinone oxidase-reductases are key enzymes involved in oxidation–reduction reactions of quinones for cellular energy generation. Quinones are substrates of flavin enzymes and are present in all prokaryotes and eukaryotes. During oxidation–reduction reactions, quinones are reduced either to semi-quinones or hydro-quinones, the latter being more stable and non-toxic (Elanskaya et al. 2004) hinting on the role of NADPH-quinone oxidase-reductases in quinone detoxification. Adenosine triphosphate (ATP)-synthase CF1 alpha subunit is directly related to energy generation in the form of ATP

(Kohzuma et al. 2009). Up-regulation of this enzyme was indicative of the improved energy metabolism and growth state in the treated seedlings.

Enhanced level of enzymes related to protein synthesis or linked processes, for example, muturase K, G-box binding factor 2 (GBF2), putative polynucleo adenyltransferase, and RNA helicase was also noticed. Up-regulation of the enzymes related to protein synthesis or linked processes points toward the fact that protein synthesis was on peak in the fungal CF treated seedlings, to fulfill proteins requirements (e.g. enzymes or structural) of the actively dividing and expanding cells. Up-regulation of transport protein such as K transporter putative was also recorded in treated samples, suggesting enhanced uptake of minerals from soil to support active growth and development.

In current study, an increased level of cobalamine independent methionine synthase was observed in the fungal CF treated plants. These enzymes are required for the regeneration of the methyl group of S-adenosyl-L-methionine, a methyl donor for several biological methylation reactions (Eichel et al. 1995). Expression of microspores specific WRKY34 was enhanced by more than 2-fold in treated plant samples. The expression of WRKY34 in *Arabidopsis* was shown to be male gametophyte specific, involved in pollen development (Zou et al. 2010). Similarly, the role of GAs has been implicated in pollen viability and development (Chhun et al. 2007), linking the activity of this hormone with WRKY34.

Conclusion

The fungal culture filtrate influenced *G. littoralis* by modulating the expression of multiple proteins. These proteins have been reported previously for their roles in a variety of biological processes, suggesting that plant growth is the result of intricate interactions of a large number of proteins linked in a complex dynamic network. Current study also highlighted an enhanced hormonal crosstalk to induce plant growth in *P. citrinum* KACC43900 treated plants.

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References

Alcazar R, Altabella T, Marco F, Bortolotti C, Reymond M, Koncz C, Carrasco P, Tiburcio AF. 2010. Polyamines: molecules with regulatory functions in plant abiotic stress tolerance. Planta. 231:1237–1249.

Baek D, Jin Y, Jeong JC, Lee HJ, Moon H, Lee J, Shin D, Kang CH, Kim DH, Nam J. 2008. Suppression of reactive oxygen species by glyceraldehyde-3-phosphate dehydrogenase. Phytochemistry. 69:333–338.

Bradford MM. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem. 72:248–254.

Chhun T, Aya K, Asano K, Yamamoto E, Morinaka Y, Watanabe M, Kitano H, Ashikari M, Matsuoka M, Ueguchi-Tanaka M. 2007. Gibberellin regulates pollen viability and pollen tube growth in rice. Plant Cell. 19:3876–3888.

Choe HT, Cosgrove DJ. 2004. Expansins as agents in hormone action. In: Davies PJ, editor. Plant hormones: biosynthesis, signal transduction, action. Dordrecht: Kluwer; p. 262–281.

Cohen E, Arad SM, Heimer YM, Mizrahi Y. 1982. Participation of ornithine decarboxylase in early stages of tomato fruit development. Plant Physiol. 70:540–543.

Depuydt S, Hardtke CS. 2011. Hormone signalling crosstalk in plant growth regulation. Curr Biol. 21:R365–R373.

Doty SL. 2011. Growth-promoting endophytic fungi of forest trees. Endophytes Forest Trees. 80:151–156.

Eichel J, Gonzalez JC, Hotze M, Matthews RG, Schröder J. 1995. Vitamin-B12-independent methionine synthase from a higher plant (*Catharanthus Roseus*). Eur J Biochem. 230:1053–1058.

El-Tarabily KA, Nassar AH, Hardy GESJ, Sivasithamparam K. 2009. Plant growth promotion and biological control of *Pythium aphanidermatum*, a pathogen of cucumber, by endophytic actinomycetes. J Appl Microbiol. 106: 13–26.

Elanskaya IV, Grivennikova VG, Groshev VV, Kuznetsova GV, Semina ME, Timofeev KN. 2004. Role of NAD (P) H: quinone oxidoreductase encoded by drgA gene in reduction of exogenous quinones in cyanobacterium *Synechocystis* sp. PCC 6803 cells. Biochemistry (Moscow). 69:137–142.

Ferreira S, Hjernã, K, Larsen M, Wingsle G, Larsen P, Fey S, Roepstorff P, Pais MS. 2006. Proteome profiling of *Populus euphratica* Oliv. upon heat stress. Ann Bot. 98:361–377.

Gamalero E, Glick BR. 2011. Mechanisms used by plant growth-promoting bacteria. In: Maheshwari DK, editor. Bacteria in agrobiology: plant nutrient management. Berlin-Heidelberg: Springer; p. 17–46.

Gonzalez E, Delsol MA. 1981. Induction of glyconeogenic enzymes by Gibberellin A_3 in endosperm of castor bean seedlings. Plant Physiol. 67:550–554.

Hatta R, Goto M, Matsuoka T, Imai S, Watanabe K. 1967a. Studies on cultivation of *Glehnia littoralis* Fr. Schmidt Part 1. A fundamental investigation. Takeda Kenkyusho Nempo. 26:84–97.

Hatta R, Goto M, Matsuoka T, Nagao Y, Okumura Y. 1967b. Studies on cultivation of *Glehnia littoralis* Fr. Schmidt Part 2. Studies on the comparison between two Strains. Takeda Kenkyusho Nempo. 26:98–106.

Hoagland DR, Arnon DI. 1950. The water-culture method for growing plants without soil. Circular 347. 2nd ed. Berkeley (CA): California Agricultural Experiment Station.

Hussain A, Hasnain S. 2011. Interactions of bacterial cytokinins and IAA in the rhizosphere may alter phytostimulatory efficiency of rhizobacteria. W J Microbiol Biotechnol. 27:2645–2654.

Jaillais Y, Belkhadir Y, Balsemã£o-Pires E, Dangl JL, Chory J. 2011. Extracellular leucine-rich repeats as a platform for receptor/coreceptor complex formation. Proc Natl Acad Sci U S A. 108:8503–8507.

Jamet E, Albenne C, Boudart G, Irshad M, Canut H, Pont-Lezica R. 2008. Recent advances in plant cell wall proteomics. Proteomics. 8:893–908.

Jewett MC, Hofmann G, Nielsen J. 2006. Fungal metabolite analysis in genomics and phenomics. Curr Opin Biotechnol. 17:191–197.

Jiang Q, Chen H, Pan X, Pan Q, Shi Y, Li X, Zhang G, Wang Y, Xie S, Shen S. 2008. Proteomic analysis of wheat (*Triticum aestivum* L.) hybrid necrosis. Plant Sci. 175:394–401.

Joyard J, Ferro M, Masselon C, Seigneurin-Berny D, Salvi D, Garin J, Rolland N. 2010. Chloroplast proteomics

highlights the subcellular compartmentation of lipid metabolism. Prog Lipid Res. 49:128–158.
Kadis C, Georghiou K. 2010. Seed dispersal and germination behavior of three threatened endemic labiates of Cyprus. Plant Sp Biol. 25:77–84.
Kagawa T, Gonzalez E. 1981. Organelle-specific isozymes of citrate synthase in the endosperm of developing Ricinus seedlings. Plant Physiol. 68:845–850.
Khan AL, Waqas M, Hussain J, Al-Harrasi A, Al-Rawahi A, Al-Hosni K, Kim MJ, Adnan M, Lee IJ. 2014. Endophytes *Aspergillus caespitosus* LK12 and *Phoma* sp. LK13 of *Moringa peregrina* produce gibberellins and improve rice plant growth. J Plant Interact. 9:731–737.
Khan SA, Hamayun M, Yoon H, Kim HY, Suh SJ, Hwang SK, Kim JM, Lee IJ, Choo YS, Yoon UH. 2008. Plant growth promotion and *Penicillium citrinum*. BMC Microbiol. 8:231.
Kohzuma K, Cruz JA, Akashi K, Hoshiyasu S, Munekage YN, Yokota A, Kramer DM. 2009. The long-term responses of the photosynthetic proton circuit to drought. Plant Cell Environ. 32:209–219.
Koyama H, Kawamura A, Kihara T, Hara T, Takita E, Shibata D. 2000. Overexpression of mitochondrial citrate synthase in *Arabidopsis thaliana* improved growth on a phosphorus-limited soil. Plant Cell Physiol. 41:1030–1037.
Kunkel G. 1984. Plants for human consumption: an annotated checklist of the edible phanerogams and ferns. University of Minnesota: Koeltz Scientific Books; p. 393.
Lin Z, Ni Z, Zhang Y, Yao Y, Wu H, Sun Q. 2005. Isolation and characterization of 18 genes encoding Î±- and Î2-expansins in wheat (*Triticum aestivum* L.). Mol Genet Genom. 274:548–556.
Liu Q, Hui H, Liu M. 2004. Determination of seed vitality and germination rate of the endangered plant *Glehnia littoralis*. J Plant Res Environ. 13:55–56.
Mangeon A, Magioli C, Menezes-Salgueiro AD, Cardeal V, de Oliveira C, Galvã£o VC, Margis R, Engler G, Sachetto-Martins G. 2009. At GRP5, a vacuole-located glycine-rich protein involved in cell elongation. Planta. 230:253–265.
Miller BL, Huffaker RC. 1982. Hydrolysis of ribulose-1, 5-bisphosphate carboxylase by endoproteinases from senescing barley leaves. Plant Physiol. 69:58–62.
Mineo L. 1990. Plant tissue culture techniques. Tested studies in laboratory teachings. Proc Able. 11:151–174.
Mitchum MG, Yamaguchi S, Hanada A, Kuwahara A, Yoshioka Y, Kato T, Tabata S, Kamiya Y, Sun T. 2006. Distinct and overlapping roles of two gibberellin 3-oxidases in Arabidopsis development. Plant J. 45:804–818.
Miyazawa M, Kurose K, Itoh A, Hiraoka N. 2001. Comparison of the essential oils of *Glehnia littoralis* from Northern and Southern Japan. J Agric Food Chem. 49:5433–5436.
Portis AR. 2003. Rubisco-activase Rubisco's catalytic chaperone. Photosynth Res. 75:11–27.
Richardson AE, Barea JM, McNeill AM, Prigent-Combaret C. 2009. Acquisition of phosphorus and nitrogen in the rhizosphere and plant growth promotion by microorganisms. Plant Soil. 321:305–339.
Santner A, Calderon-Villalobos LIA, Estelle M. 2009. Plant hormones are versatile chemical regulators of plant growth. Nat Chem Biol. 5:301–307.
Scheler C, Lamer S, Pan Z, Li XP, Salnikow J, Jungblut P. 1998. Peptide mass fingerprint sequence coverage from differently stained proteins on two-dimensional electrophoresis patterns by matrix assisted laser desorption/ionization mass spectrometry (MALDI-MS). Electrophoresis. 19:918–927.
She J, Han Z, Kim TW, Wang J, Cheng W, Chang J, Shi S, Wang J, Yang M, Wang ZY. 2011. Structural insight into brassinosteroid perception by BRI1. Nature. 474:472–476.
Shen S, Matsubae M, Takao T, Tanaka N, Komatsu S. 2002. A proteomic analysis of leaf sheaths from rice. J Biochem. 132:613–620.
Shevchenko A, Wilm M, Vorm O, Mann M. 1996. Mass spectrometric sequencing of proteins from silver-stained polyacrylamide gels. Anal Chem. 68:850–858.
Sirover MA. 2005. New nuclear functions of the glycolytic protein, glyceraldehyde-3-phosphate dehydrogenase, in mammalian cells. J Cell Biochem. 95:45–52.
Tsugita A, Kamo M. 1999. 2-D electrophoresis of plant proteins. Methods Mol Biol. 112:95–97.
Vanhaeren H, Gonzalez N, Coppens F, Milde LD, Van Daele T, Vermeersch M, Eloy N, Storme V, Inze D. 2014. Combining growth-promoting genes leads to positive epistasis in Arabidopsis thaliana. 3: e02252. Available from: http://dx.doi.org/10.7554/eLife.02252
Waqas M, Khan AL, Lee IJ. 2014. Bioactive chemical constituents produced by endophytes and effects on rice plant growth. J Plant Interact. 9:478–487.
Werck-Reichhart D, Hehn A, Didierjean L. 2000. Cytochromes P450 for engineering herbicide tolerance. Trends Plant Sci. 5:116–123.
Westermeier R. 2006. Electrophoresis in practice: a guide to methods and applications of DNA and protein separations. Weinheim: Wiley-VCH Verlag GmbH & Co. KGaA.
Woo HH, Jeong BR, Hirsch AM, Hawes MC. 2007. Characterization of Arabidopsis AtUGT85A and AtGUS gene families and their expression in rapidly dividing tissues. Genomics. 90:143–153.
Wu CH, Bernard SM, Andersen GL, Chen W. 2009. Developing microbe–plant interactions for applications in plant growth promotion and disease control, production of useful compounds, remediation and carbon sequestration. Microb Biotechnol. 2:428–440.
Xu M, Wilderman PR, Peters RJ. 2007. Following evolution's lead to a single residue switch for diterpene synthase product outcome. Proc Natl Acad Sci U S A. 104:7397–7401.
Yu-ling Z. 2008. Study on seed quality detection and quality standard of *Glehnia littoralis* Schmidt. J Anhui Agric Sci. 23:78–82.
Zou C, Jiang W, Yu D. 2010. Male gametophyte-specific WRKY34 transcription factor mediates cold sensitivity of mature pollen in Arabidopsis. J Exp Bot. 61:3901–3914.