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Changes in the 2DE protein profiles of chilli pepper (*Capsicum annuum*) leaves in response to *Fusarium oxysporum* infection

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ABSTRACT: Wilt disease caused by *Fusarium oxysporum* f. sp. *capsici* is a major problem of chilli pepper production worldwide that calls for a better understanding of defensive mechanisms in the chilli plant. We used a proteomic technique to investigate protein responses of chilli pepper to *F. oxysporum* f. sp. *capsici*. Two cultivars of resistant (Mae Ping 80) and susceptible (Long Chilli 455) plants were cultured in vitro. Chilli plants at 6-week growth were then infected with a suspension of *F. oxysporum* f. sp. *capsici* or distilled water used as a control. After 48 h of infection, proteins were extracted and analysed using 2DE to identify the responsive proteins. At least 9 spots were differentially expressed in the resistant cultivar (5 increasing, 4 decreasing) and 1 supplementary; while 15 increasing, 11 decreasing, and 11 supplementary protein spots were found in the susceptible cultivar. These proteins were then identified by MALDI-TOF MS combined with bioinformatics methods. Some of the induced proteins e.g., NADPH HC toxin reductase, serine/threonine protein kinase, and 1-aminocyclopropane-1-carboxylate synthase 3 are involved in plant defence mechanism. In order to determine the *Fusarium* wilt protective proteins in chilli plant, the protein patterns of healthy resistance were compared with those of susceptible cultivars. Interestingly, resistance showed higher expression of proteins related to ROS detoxification. Moreover, the ability of chilli plant to resist *Fusarium* wilt disease was related to the expression of non-inducible immunity 1 protein.

KEYWORDS: *Fusarium* wilt, PR-proteins, plant-pathogen interactions

INTRODUCTION

Chilli pepper (*Capsicum annuum* L.) is widely cultivated and used all over the world. The *Fusarium* wilt disease, caused by the soil-borne fungus, *Fusarium oxysporum* is a serious disease that reduces growth, fruit yield, and quality, threatening chilli production. This fungus enters the vascular systems via the root tissues and subsequently uses the xylem vessels as avenues to rapidly colonize the plant, leading to the characteristic wilt symptoms[1]. *F. oxysporum* is difficult to control as it survives in field soil for several years. Commercial fungicides used to control outbreaks of the *Fusarium* wilt disease can be an environmental hazard[2]. Moreover, *F. oxysporum* species is able to detoxify fungicides by biological conversion causing fungicide resistance[3]. To avoid these problems, the biological control of plant disease is being actively pursued. The understanding of plant-pathogen interactions including plant defence responses is a challenging issue which may lead to the development of new strategies to control plant disease.

When plants are attacked by pathogens, their self-defence mechanisms, such as local signal generation, hypersensitive response (HR) activation, cell wall accumulation, phytoalexin synthesis, pathogenesis-related (PR) protein expression, and systemic resistance induction are activated[4]. The expression of PR-proteins is a significant mechanism that plants have developed to protect themselves from pathogen invasion. These PR-proteins have been classified into 17 families based on their primary structures, immunologic relationships, and enzymatic properties including chitinases (PR-3, -4, -8, -11), β-1, 3-glucanases (PR-2), proteinase inhibitors (PR-6), and peroxidases (PR-9)[5]. Introduction of PR-genes has been successfully used to improve disease resistance in some plant species[6,7].

Studies of plant-pathogen interactions have mostly focused on PR-proteins and other proteins involved in defence mechanisms. However, the mechanisms of plant defence response are controlled by multiple biochemical pathways[8]. To study the global responses in plants, proteomics has become a

powerful tool[9]. Proteomic methods consist of two techniques where proteins are first separated by two-dimensional gel electrophoresis (2DE) and interesting protein spots are then identified by mass spectrometry (MS). In contrast to mRNA expression profiling, proteomic approaches monitor the actual protein composition of the cells or a certain tissue which is directly determined by biochemical cellular pathways[10].

Proteomic studies in plants aim to identify specific proteins related to biotic and abiotic stress. The PR-10 protein has been reported as the salt-stress-responsive protein in grapevine[11] and peanut[12]. Proteins responding to salt and drought tolerance were also investigated in rice[13]. An investigation of protein response to crenate broomrape in pea using a proteomic approach has also been reported[14] and specific proteins related to bacterial infection in *Arabidopsis* have been examined[15]. A similar study on plant-pathogen interactions in legumes was later reported[16]. In addition, proteins related to pathogen infection in the chilli plant have also been analysed using a proteomic approach. Several families of PR-proteins and proteins involving programmed cell death (PCD) and a type of HR mechanism were characterized in the chilli plant induced by tobacco mosaic virus and pepper mild mottle virus[17,18]. However, proteomic studies of chilli plant-pathogen interactions are still limited.

In this study, we aim to investigate the defensive protein response to *F. oxysporum* attack in the chilli plant. For this purpose, the protein expression patterns of non-infected resistant cultivars were compared with those of susceptible cultivars. The differential expressions between infected resistant and infected susceptible cultivars were also investigated. Proteins corresponding to the infection response were further analysed using a proteomic approach including 2DE and MALDI-TOF MS.

MATERIALS AND METHODS

Chemicals and reagents

Urea, 3-[(3-cholamidopropyl) dimethylammonio]-1-propane sulphonate (CHAPS), dithiothreitol (DTT), protease inhibitor mix, immobilized pH gradient (IPG) strip (Immobiline DryStrip, pH 3–10 nonlinear, 7 cm), IPG buffer (pH 3–10 NL), 2D Clean-up kit, 2D Quant kit, and Silver staining protein kit were purchased from GE Healthcare Biosciences (Piscataway, NJ). Unless stated otherwise, all reagents and chemicals were obtained from Sigma-Aldrich.

Fungal culture

F. oxysporum f. sp. *capsici* was isolated from *C. annuum* by the Plant Pathology Research Group, Plant Protection Research and Development Office, Department of Agriculture, Ministry of Agriculture and Cooperatives, Thailand. The culture was maintained on potato dextrose agar (PDA, Himedia) at 35 °C. Routine subculture was achieved by transferring a mycelial plug onto a fresh PDA plate.

Plant materials, pathogen and inoculation procedures

The chilli (*Capsicum annuum* L.) cultivars used in this study were Mae Ping 80 (resistant line) and Long Chilli 455 (susceptible line) obtained from Known-You Seed Co. Ltd. (43 Ratchaphuek Rd., Changpuak, Muang, Chiang Mai 53000, Thailand). Seeds were surface sterilized with 70% ethanol for 5 min followed by 1% sodium hypochlorite solution containing a few drops of Tween-20 for 15 min under vacuum. Sterile seeds were then rinsed 3 times in sterilized water, blot dried with filter paper, and transferred to basal Murashige and Skoog (MS) medium. The chilli cultures were grown at 28 °C under natural light for 6 weeks.

Spore suspension of *F. oxysporum* f. sp. *capsici* was prepared from 14 day-old culture by washing and scraping the surface of colonies with sterile distilled water and a scalpel, then filtering through two layers of cheesecloth to remove mycelial fragments. The number of spores in the suspension was adjusted to 10^7 per ml following counting with a haemacytometer. *F. oxysporum* spore suspension (40 µl) was inoculated near the root/stem regions of 6 week-old chilli plantlets[19]. As a control, plantlets were mock-inoculated with an equal volume of sterile distilled water.

Disease evaluation

Fusarium wilt symptoms in chilli plantlets were evaluated in chilli cultivars with 3 independent biological replications. Each replicate consisted of 4 treatments (mock-inoculated Mae Ping 80, inoculated Mae Ping 80, mock-inoculated Long Chilli 455, and inoculated Long Chilli 455) with 10 plantlets per treatment. The disease severity in chilli plantlets was scored everyday after inoculation with *F. oxysporum* using a scale 0–6; 0 = no disease observed, 1 = slight stunting, 2 = slight stunting and chlorosis of leaves, 3 = $<$ 10% of the leaves showing chlorosis and/or 10% of the plant with wilt symptoms, 4 = $<$ 11–25% of the plant with wilt symptoms, 5 = 26–50% of the plant

with wilt symptoms, 6 = 51–100% of the plant with wilt symptoms or plant death[20]. The disease scale values were recorded for 14 days. The percentage of disease severity was calculated at 7 days post-inoculation using the formula of Song et al[21].

$$\text{Disease severity} = \frac{\sum(\text{Disease scale} \times \text{Number of plantlets infected})}{\text{Highest scale} \times \text{Total number of plantlets}}$$

Leaf harvest and protein extraction

After 48 h of inoculation, leaves were harvested (3 plantlets per treatment). Leaves were pooled and ground to a powder in liquid nitrogen. Leaf powder (200 mg) was resuspended in 200 µl lysis solution (7 M urea, 2 M thiourea, 4% w/v CHAPS and 40 mM DTT) containing 2 µl protease inhibitor mix. The suspension was centrifuged at 13 200g at 4 °C for 15 min. The supernatant was then transferred to a new microcentrifuge tube and centrifuged again to remove the remaining cell debris. The supernatant or extracts were cleaned using a 2D Clean-up kit. Protein content of the extracts was quantified using a 2D Quant kit according to the manufacturer's instructions. The extracts were then stored at −20 °C until the 2DE was performed. For the 2DE analysis, the extracts were prepared from 3 replicates with independent inoculations.

Two-dimensional gel electrophoresis (2DE)

Samples (6 µg protein) mixed with rehydration solution (7 M urea, 2 M thiourea, 2% w/v CHAPS and 0.002% w/v bromophenol blue) containing 20 mM DTT and 0.5% v/v IPG buffer pH 3–10 NL. IPG strips (7 cm, nonlinear pH 3–10) were passively rehydrated with the sample mixture for 12 h prior to the isoelectric focusing (IEF) step. Strips were then held at 150 V for 2 h in order to remove the ionic impurities. IEF was performed on an Ettan IPGphor II (GE Healthcare Biosciences) using the optimum program as follows: 300 V 1 h, 1000 V 0.5 h, 5000 V 1.3 h, and held at 5000 V for 36 min. After IEF, IPG strips were equilibrated by soaking for 15 min in SDS equilibration solution (75 mM Tris-HCl, 6 M urea, 30% v/v glycerol, 2% w/v SDS, and 0.002% w/v bromophenol blue) containing 1% w/v DTT, and then for 15 min in the solution containing 2.5% w/v iodoacetamide. Equilibrated strips were then transferred onto 12.5% v/v SDS-polyacrylamide gels (9 cm × 10 cm, 1 mm thickness) and electrophoresis was carried out in a MiniVE vertical electrophoresis system (GE Healthcare Biosciences). 2DE was run at 10 mA/gel for 15 min, followed by 20 mA/gel for about 1 h or until the dye front reached the bottom of the gel. Gels were stained using the Silver staining protein kit according to the manufacturer's protocol. The experiment was repeated three times providing three replicate protein gels for each inoculation treatment.

Image acquisition and data analysis

The stained 2DE gels were digitized using a UMAX UTA-1120 ImageScanner (GE Healthcare Biosciences, Uppsala) in transparent mode with a resolution of 300 dpi. Twelve 2DE gels from three replicates of each treatment were analysed with IMAGEMASTER 2D PLATINUM version 5.0 (GE Healthcare Biosciences, NJ, USA). After background subtraction, protein spots in gel images were automatically detected and artifact spots were eliminated through the detection parameter set up as follows: smooth 2, minimum area 5 and saliency 50. All of the protein spots were then matched individually to the spots of reference gel in the matching step. Any mis-matched spot pairs were removed and the correct pairs were restored. The protein abundances of matched spots were determined by comparing the spot volumes. To compensate for the variation of protein loading and gel staining, the volume of protein spots were calculated by IMAGEMASTER as the relative volume (%Vol), i.e., the percentage of the total volume of all the spots present in the gel.

The average relative volumes of protein spots were derived from 3 independent replicates and were then normalized in terms of ratio in order to compare the fluctuation of protein expression between two experimental treatments. The statistical analysis of the relative volume of each matched spot was accomplished using a two-sample t-test using IMAGEMASTER. The p value for the t-test value was taken from www.psychstat.missouristate.edu/introbook/tdist.htm. The protein spots with $p < 0.05$ were considered as differential expressed protein spots and some significant spots were further subjected to MS analysis.

MALDI-TOF MS and database searching

Some significant protein spots were excised from the gels and sent to Genome Institute (GI), National Centre for Genetic Engineering and Biotechnology (BIOTEC), Thailand for matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) analysis. Mass spectra of tryptic peptides were acquired on a Bruker Biflex MALDI-TOF mass spectrometer (Bruker Daltonics). This instrument was operated in positive-ion reflector mode with 500 shots of 337 nm N_2 laser for ion

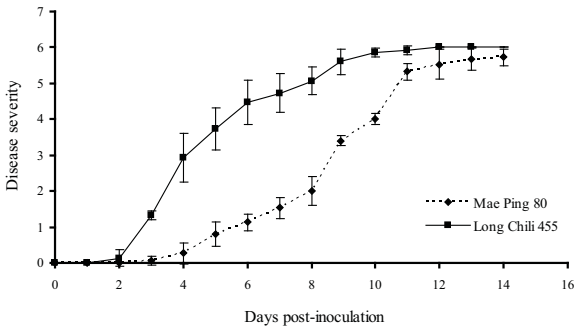

Fig. 1 Disease severity of two chilli cultivars after inoculation with *F. oxysporum*. Each bar represents a mean ± standard deviations from three independent replicates.

generation. The raw data was processed by FLEX ANALYSIS software (Bruker Daltonics) to generate the peak lists of peptide mass fingerprint (PMF). The obtained PMF of each spot was matched against the National Centre for Biotechnology Information (NCBI) database using MASCOT search engine (http://www.matrixscience.com). The following parameters were used for database searches: taxonomy restrictions to Viridiplantae (green plants), trypsin as digesting enzyme with 1 missed cleavage allowed, carbamidomethylation of cysteine as fixed modification, and oxidation of methionine as variable modification, ± 1.2 Da peptide tolerance, MH^+ and monoisotopic mass values. A Mowse score cut-off greater than 69 was qualified as a statistical significant ($p < 0.05$) for protein identification.

RESULTS

Evaluation of disease severity in chilli plants

To determine the resistance and susceptibility of chilli cultivars used in this experiment, the disease severities of *C. annuum* cv. Mae Ping 80 and cv. Long Chilli 455 were evaluated by scoring the appearance of *Fusarium* wilt disease for 14 days after inoculation. The disease severity scores of these two cultivars were proportional to the time after inoculation (Fig. 1). It is clear that the progress of the disease in Mae Ping 80 was slower than in Long Chilli 455. At 7 days after inoculation, the disease severities of Mae Ping 80 and Long Chilli 455 were 26 ± 5% and 79 ± 9%, respectively. These results suggested that Mae Ping 80 was more resistant to *Fusarium* wilt disease than Long Chilli 455. Therefore, in this experiment, Mae Ping 80 and Long Chilli 455 were classified as resistant and susceptible cultivars, respectively.

Table 1 Protein spots observed in the differential expression changes in leaves of *C. annuum* cv. Mae Ping 80 responding to *F. oxysporum* infection.

Spot no.	pI	Mw (kDa)	Quantity ratio[a] infected/healthy
1	5.82	57.1	1.23
2	5.85	56.7	1.20
3	5.93	46.4	1.17
4	6.41	26.2	1.21
5	6.64	29.8	1.20
6	5.44	28.9	0.89
7	5.49	27.6	0.90
8[b]	6.27	25.2	0.53
9	6.27	24.4	0.84
10	5.83	55.7	-[c]

[a] Quantity ratio of each spot was derived from the normalization of average relative volume from three independent replicates.
[b] Protein spots that were further analysed by MALDI-TOF MS.
[c] Not found in healthy, non-infected, Mae Ping 80.

2DE and MALDI-TOF MS analysis

The protein patterns of *C. annuum* cv. Mae Ping 80 and cv. Long Chilli 455 were examined at 48 h after inoculation with *F. oxysporum*. 6 µg of leaf proteins were separated by 2DE. After silver staining, gels were compared in three groups as follows: (1) healthy and infected Mae Ping 80, (2) healthy and infected Long Chilli 455, and (3) healthy Mae Ping 80 and healthy Long Chilli 455. The significant protein spots which were differentially expressed, including newly detected ones, are shown in Figs. 2–4. Most of the protein spots that showed significant changes in protein expression had isoelectric points (pI) and molecular weights (Mw) within 4–7 and 13–70 kDa, respectively. Lists of pI, Mw, and quantity ratio of differentially expressed protein spots are shown in Table 1–3. Some significant spots were selected for MALDI-TOF MS analysis. These proteins were identified by comparing their PMF with the proteins in the database using MASCOT software. Matched protein spots are listed in Table 4.

Protein changes in resistant cultivars after *F. oxysporum* infection

The changes of protein level in the resistant cultivars, Mae Ping 80, were investigated at 48 h after inoculation by comparing the 2DE gel of infected with non-infected leaf proteins. The results revealed that 10 protein spots were quantitatively and qualitatively different in protein abundances with 5 increasing, 4

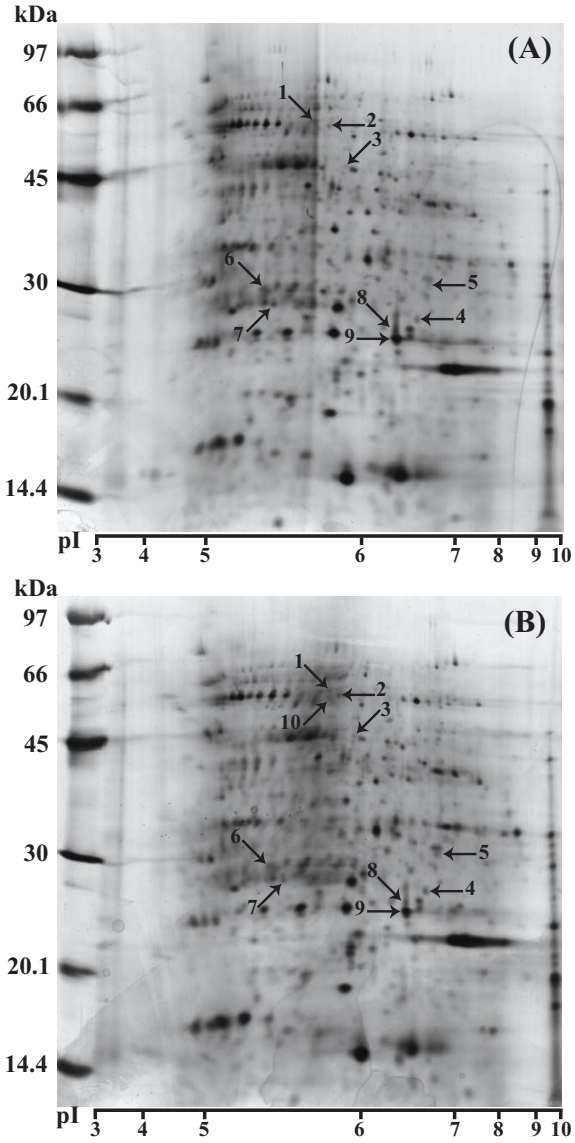

Fig. 2 Comparison of silver-stained 2DE gel between (A) healthy and (B) infected *C. annuum* cv. Mae Ping 80 (resistant cultivars). Protein spots that showed expression-level changes are indicated by arrows and their pI and Mw are displayed in Table 1.

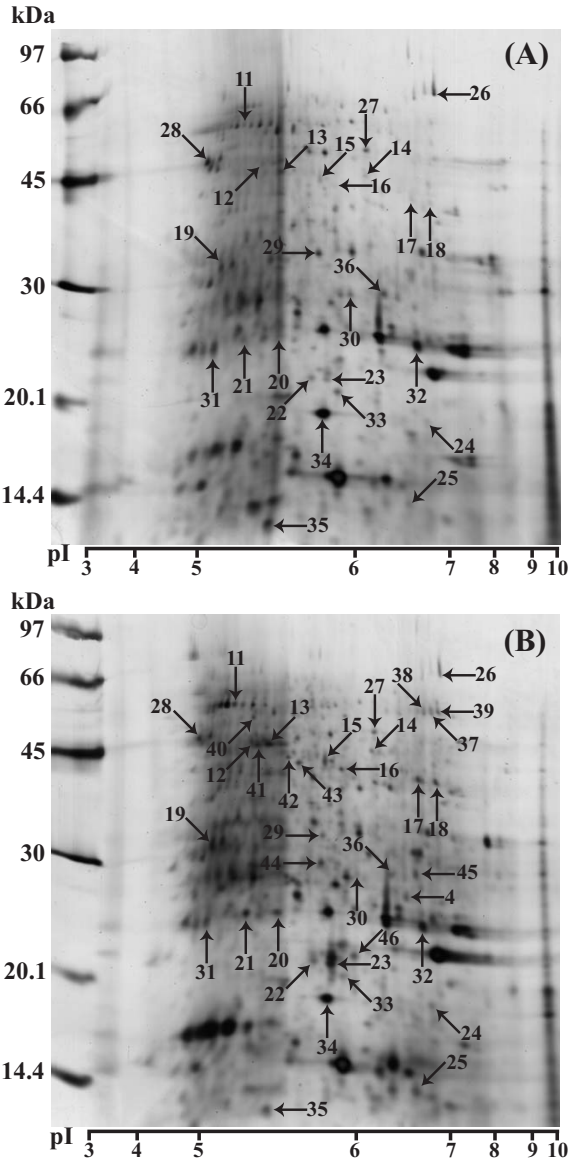

Fig. 3 Comparison of silver-stained 2DE gel between (A) healthy and (B) infected *C. annuum* cv. Long Chilli 455 (susceptible cultivars). Protein spots that showed expression-level changes are indicated by arrows and their pI and Mw are displayed in Table 2.

decreasing, and 1 newly induced (Fig. 2 and Table 1). Spot no. 8 which showed a decrease after infection was further analysed and identified as peroxidase 2 (fragment). Peroxidase is an enzyme classified in the pathogenesis-related (PR) protein family[5].

Protein changes of susceptible cultivars after *F. oxysporum* infection

The protein spots in 2DE gels of infected susceptible cultivars, Long Chilli 455, were compared with that of healthy cultivars to determine changes in expression level. Interestingly, the number of differentially expressed protein spots in susceptible cultivars was higher than the changes in resistant cultivars.

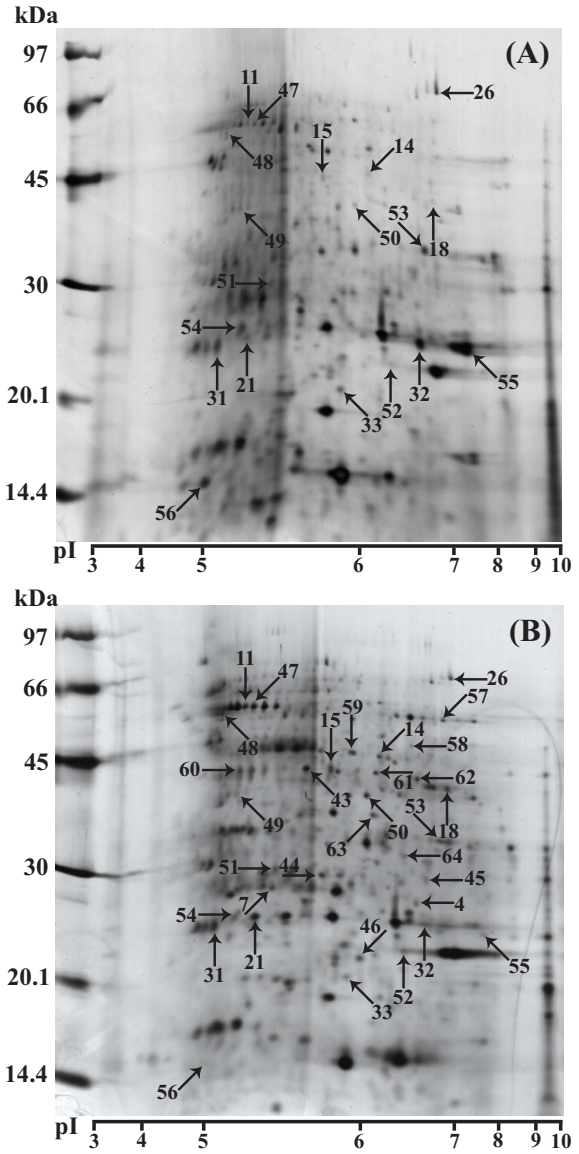

Fig. 4 Silver-stained 2DE gel of (A) healthy *C. annuum* cv. Long Chilli 455 and (B) cv. Mae Ping 80. Protein spots that showed expression-level changes are indicated by arrows and their pI and Mw are displayed in Table 3.

Table 2 Protein spots observed in the differential expression changes in leaves of *C. annuum* cv. Long Chilli 455 responding to *F. oxysporum* infection.

Spot no.	pI	Mw (kDa)	Quantity ratio[a] infected/healthy
4[b]	6.41	26.2	-[c]
11[b]	5.25	57.7	1.31
12	5.40	47.7	4.38
13	5.52	47.2	1.51
14	6.10	45.3	1.16
15	5.84	43.5	1.16
16	5.91	42.6	1.22
17	6.56	40.6	1.79
18[b]	6.90	40.2	1.49
19	5.04	31.4	1.42
20	5.54	24.4	1.63
21[b]	5.33	24.3	5.40
22	5.77	21.1	2.22
23	5.87	20.7	1.29
24	6.75	18.2	1.40
25	6.58	13.7	1.26
26	6.96	69.7	0.80
27	6.13	50.4	0.74
28	4.88	48.3	0.59
29	5.82	32.4	0.61
30	6.01	28.1	0.53
31	4.94	23.8	0.54
32[b]	6.66	23.8	0.83
33	5.94	19.9	0.59
34	5.85	18.4	0.58
35	5.51	14.2	0.58
36	6.26	28.2	0.82
37[b]	6.75	55.6	-
38	6.62	55.6	-
39	6.88	56.0	-
40	5.38	54.5	-
41	5.43	46.5	-
42	5.59	43.9	-
43	5.67	43.0	-
44[b]	5.81	29.1	-
45	6.52	28.4	-
46	6.01	21.0	-

[a] Quantity ratio of each spot was derived from the normalization of average relative volume from three independent replicates.

[b] Protein spots that were further analysed by MALDI-TOF MS.

[c] Not found in healthy, non-infected, Long Chilli 455.

In susceptible cultivars there were 37 protein spots showing significant changes in expression level with 15 increasing, 11 decreasing, and 11 newly detected (Fig. 3 and Table 2). Seven protein spots were chosen for further analysis and then identified. However, only 4 protein spots were characterized. Proteins with induced expression after infection were identified as ATP synthase CF1 β-subunit and NADPH HC toxin reductase (HCTR). These proteins function in ATP production and toxin detoxification, respectively. Two new protein spots detected after infection were also identified as serine/theronine (Ser/Thr) protein kinase and 1-aminocyclopropane-1-carboxylate (ACC) synthase 3. These proteins are involved in protein phosphorylation and ethylene biosynthesis, respectively.

Table 3 Protein spots that showed qualitative or quantitative changes in leaves of healthy *C. annuum* cv. Mae Ping 80 (resistant) compared with Long Chilli 455 (susceptible).

Spot no.	pI	Mw (kDa)	Quantity ratio[a] Mae Ping/long Chilli
4[b]	6.41	26.2	-[c]
7	5.49	27.6	-
11[b]	5.25	57.7	1.45
14	6.10	45.3	1.96
15	5.84	43.5	1.19
18[b]	6.90	40.2	1.22
21[b]	5.33	24.3	3.36
26	6.96	69.7	0.72
31	4.94	23.8	0.78
32[b]	6.66	23.8	0.52
33	5.94	19.9	0.66
43	5.67	43.0	-
44[b]	5.81	29.9	-
45	6.52	28.4	-
46	6.01	21.0	-
47[b]	5.33	57.5	1.27
48	5.03	55.8	3.44
49	5.18	39.3	1.22
50[b]	6.03	39.0	1.60
51	5.51	29.7	1.78
52	6.30	21.9	1.62
53	6.98	69.0	0.90
54	5.20	25.1	0.72
55	7.70	23.5	0.27
56	4.77	14.7	0.86
57	6.83	54.0	-
58	6.35	47.5	-
59	5.92	46.3	-
60	5.19	42.2	-
61[b]	6.07	43.0	-
62	6.41	41.5	-
63[b]	6.07	36.2	-
64	6.29	31.0	-

[a] Quantity ratio of each spot was derived from the normalization of average relative volume from three independent replicates.
[b] Protein spots that were further analysed by MALDI-TOF MS.
[c] Not found in healthy, non-infected, Long Chilli 455.

Protein changes among healthy resistant and susceptible cultivars

The protein patterns of healthy resistant cultivars were compared with those of susceptible cultivars in order to examine the protective proteins in chilli. The expression of 19 protein spots changed (11 increases, 8 decreases) and 14 protein spots were only found in resistant cultivars (Fig. 4 and Table 3). Ten protein spots were excised, analysed and identified. Four proteins significantly matched to proteins in the database. Two protein spots which showed the higher expression were identified as ATP synthase CF α-subunit, which function in ATP production, and putative copper/zinc superoxide dismutase (Cu/Zn SOD) which is involved in reactive oxygen species (ROS) detoxification. Interestingly, two protein spots present only in the resistant cultivars were involved in plant disease resistance (non-inducible immunity 1 (NIM1) and disease resistance protein A19).

DISCUSSION

When a plant is attacked by a pathogen, a complex network of plant defence mechanisms are brought into play to protect the plant from damage. Many techniques have been developed to monitor the changes of these mechanisms. Proteomics offers a variety of powerful techniques to study the alteration of plant defence mechanism at the protein level. In this study, we used a proteomic technique to identify proteins responding to *F. oxysporum* in *Capsicum annuum* by comparing the protein patterns of healthy plants with those of infected plants. We also examined pathogen protective proteins in chilli plants by comparing the protein patterns of resistant cultivars with those of susceptible cultivars. Mae Ping 80 and Long Chilli 455 are resistant and susceptible cultivars, respectively. This was confirmed by comparing disease severity of these cultivars after being infected with *F. oxysporum*[21].

To investigate *F. oxysporum* defensive proteins in the chilli plant, we examined protein responses 48 h after infection due to the early defence against pathogen previously reported[17,22,23]. Leaves were chosen as the source of protein extraction as previously reported since apoplastic fluid or xylem sap of infected plants is a rich source to study plant defensive proteins[8]. Protein extracts were separated by 2DE. The differential expression of protein profiles between healthy and infect plants were then analysed. Most of protein spots responding in susceptible plants were also found in resistant plants suggesting that some defensive proteins are already present in resistant plants. Thus minor changes of protein expression in the resistant plant were observed. Similar evidence was also reported earlier in resistant cultivars of pea in response to powdery mildew fungus, *Erysiphe pisi* in comparison to the susceptible cultivars[24].

The combination of MALDI-TOF MS analysis and NCBI database searching allowed the identification of 9 protein spots. Three identified proteins including peroxidase 2 (spot no. 8), Ser/Thr protein

Table 4 PMF analysis and identification of differentially expressed protein in chilli leaves.

Spot no.	Accession no.[a]	Protein identity	Species	%Sequence coverage[b]	Queries matched[c]	pI/Mw(kDa)[d]	
						Theoretical	Experimental
8	gi|464361	Peroxidase 2 (fragment)	*Hordeum vulgare*	67	16	10.17/19.0	6.27/25.2
11	gi|118614499	ATP synthase CF1 β-subunit	*Sorghum bicolor*	28	8	5.31/54.0	5.25/57.7
18	gi|2911360	NADPH HC toxin reductase	*Zea mays*	47	17	5.88/39.0	6.90/40.2
37	gi|38228677	Serine/Threonine protein kinase	*Fagus sylvatica*	59	20	5.56/41.7	6.75/55.6
44	gi|62529137	1-aminocyclopropane--1-carboxylate synthase 3	*Rosa* hybrid cultivar	61	19	8.37/21.4	5.81/29.1
47	gi|89280620	ATP synthase CF1 α-subunit	*Solanum lycopersicum*	28	12	5.14/55.4	5.33/57.5
50	gi|3108347	Putative copper/zinc superoxide dismutase	*Arabidopsis thaliana*	41	17	5.37/27.7	6.03/39.0
61	gi|49182274	Non-inducible immunity 1	*Lycopersicon esculentum*	54	37	5.94/65.0	6.07/43.0
63	gi|6648977	Disease resistance protein A19	*Capsicum annuum*	50	20	7.72/24.2	6.07/36.2

[a] Accession number of proteins derived from NCBI database using MASCOT search.
[b] Percentage of sequence coverage calculated from sequence of matched amino acid/total amino acid × 100.
[c] Number of searched peptides matched with peptides in database.
[d] pI and Mw (kDa) values calculated from amino acid sequence of protein in database (theoretical) and the position of protein spots in 2DE gel using IMAGEMASTER 2D PLATINUM version 5.0.

kinase (spot no. 37), and Cu/Zn SOD (spot no. 50) corresponded to proteins involved in signalling pathways in plant defence mechanism, for example, the production of ROS and nitric oxide (NO), activation of mitogen-activated protein kinase (MAPK), fluxes in calcium and other ions, and changes in defensive gene expression. ROS including superoxide anion (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radical ($OH\cdot$), are generated by the sequential reduction of molecular oxygen through the mechanisms of glutathione, superoxide dismutase (SOD), peroxidase, and catalase. Although these oxygen species primarily occur at a minimal level in the plant, their levels are raised within 2–3 min after pathogen attack or elicitor treatment. The accumulation of ROS is one part of the signalling pathway that induces plant defence mechanisms[4].

Hydrogen peroxide, a member of ROS, is known as a signal to trigger PCD causing localized cell death which can prevent pathogen outbreak to other cells[25]. A high level of H_2O_2 within plant cells also activates a process of the MAPK which leads to changes of defensive gene expression and other cellular processes[26]. In infected resistant cultivars, we observed the decreased expression of peroxidase 2. The enzyme catalyses the conversion of H_2O_2 to water via the reduction of oxygen. This result indicates the accumulation of H_2O_2 which may be crucial for *Fusarium* resistance through the PCD and MAPK cascade. The lower expression level of peroxidase 2 was also described in *Fusarium* head blight infected wheat spike[27] and *Fusarium* elicitor treated *Arabidopsis* cell culture[28].

SOD (EC 1.15.1.1) is also involved in the ROS detoxification. This metalloenzyme protects plant cells from the effect of ROS through catalysing the conversion of O_2^- to a signal molecule H_2O_2[29]. The high abundance of protein spot detected in resistant plants corresponds to putative Cu/Zn SOD. The increment of SOD expression may contribute to the defence responses and resistance mechanisms in resistant cultivars. The investigation of the *Brassica* crop proteome suggested that SOD may be involved in the detoxification of superoxide radicals generated during fungal infection[30]. Moreover, a previous report demonstrated that the transgenic potato conferring lily Cu/Zn-SOD gene elevated the resistance against the bacterial pathogen *Erwinia carotovora* through the overexpression of SOD enzyme activity which subsequently decreased the number of superoxide radicals[31].

Protein kinases have an important function in signal transduction during pathogen infection and subsequent induction of plant defence mechanisms. A typical protein kinase such as serine/threonine MAP kinase can activate the MAPK cascade, a key signalling pathway for disease resistance, through the phosphorylation of hydroxyl groups on serine and threonine residues. The reversible phosphorylation and dephosphorylation in MAPK cascade often serves as an on-off switch for regulation of cellular activities[4,32]. In our study, the protein spot no. 37 identified as Ser/Thr protein kinase was newly induced in susceptible cultivars after fungal infection. Early research revealed that the *Pto* gene which encodes a tomato Ser/Thr protein kinase can confer a resistance to the bacterial pathogen *Pseudomonas syringae*[33]. Therefore, the induction of Ser/Thr protein kinase

expression in chilli plant possibly activates the MAPK via phosphorylation process which then leads to the sequential defence mechanisms.

Protein spot no. 44 which was detected in susceptible chilli plants upon *F. oxysporum* infection was similar to ACC synthase 3 (spot no. 44). The activities of ACC synthase (EC 4.4.1.14) and ACC oxidase (EC 1.14.17.4) are responsible for ethylene (ET) biosynthesis[34]. ET is a plant hormone that plays a role in the regulation of plant developmental processes such as seed germination, flower and leaf senescence, fruit ripening, leaf abscission, and is also responsible for environmental stress and pathogen invasion[35]. The accumulation of ACC synthase in the plant may be the reason susceptible plants showed higher severity of wilt symptoms than resistant plants. This explanation was supported by Lund et al[36] who found that the wilt symptoms of the tomato mutant with an impairment of ET synthesis were significantly reduced. ET also regulates the MAPK cascade via the constitutive triple response 1 (CTR1) protein which triggers the activation of different defence mechanisms in plants[37]. Thus the expression of ACC synthase 3 in infected chilli may contribute to the MAPK cascade, the prior defence response, through ET biosynthesis. A proteomic study of cucumber and pumpkin also reported the induction of ACC synthase expression[38].

Two protein spots (spot no. 11 and 47) were identified as ATP synthase CF1 β-subunit and α-subunit, respectively. ATP synthase is involved in photosynthesis by storing the energy derived from a light reaction within the chloroplast via the production of ATP from ADP and inorganic phosphate in the presence of a proton gradient[39]. The expression of ATP synthase suggests that chilli plants may require the energy to promote enzyme activities and protein synthesis to defend against the invasion of fungi. Higher expression of ATP synthase α- and β-subunit were previously described in a rice mutant proteome[40]. Interestingly, the abundance of ATP synthase CF1 was increased in infected susceptible cultivars. In contrast, such evidence was not observed in infected resistant plants. In healthy plants, the relative volume of ATP synthase CF1 in resistant cultivars was higher than that for susceptible cultivars. This suggests that the accumulation of ATP generated from this enzyme may be necessary for multiple defence mechanisms in susceptible plants after pathogen infection. In addition, the expression of ATP synthase CF1 is possibly related to the resistant ability as this protein was present at a high level in non-infected resistant plants.

Regarding plant defence mechanisms, enzymes in the reductase family have been reported to confer the ability to detoxify the toxins produced by microorganisms[41]. For example, HCTR, encoded by the *Hm1* gene, was activated to detoxify the HC-toxin generated by the fungal pathogen *Cochliobolus carbonum* causing wilt and necrosis symptoms in maize leaf. This enzyme activates the conversion of HC-toxin into the inactive form through the reduction of an 8-keto group[42]. *F. oxysporum* can also produce the HC-toxin like structure compound known as enniatin toxin which can trigger wilt disease in the host plants[43]. In non-infected chilli plants, the abundance of protein corresponding to HCTR (spot no. 18) in resistant cultivars was higher than in the susceptible cultivar. After *F. oxysporum* infection, a higher expression level of this protein was observed in susceptible cultivars but not in resistant cultivars. These results indicated that resistant cultivars may be capable of detoxification. Therefore, the assistance of HCTR enzymes is not necessary. Previous studies have shown that the high expression of HCTR is related to the resistance of many plants to both biotic and abiotic stresses. Uhimiya et al[44] demonstrated that the transgenic rice overexpressing *YK1* gene, encoding the protein HCTR-like activity, was resistant to rice blast disease caused by *Magnaporthe grisea*. This transgenic plant also exhibited high tolerance to several abiotic stresses such as NaCl, UV-C, submergence and H_2O_2. Furthermore, *Hm1* gene regulation of HCTR biosynthesis responding to H_2O_2 stress tolerance in maize was reported[45]. Therefore, the abundance of HCTR in Mae Ping 80 may be the key to why this cultivar is more resistant than Long Chilli 455.

Defence mechanisms such as the processes of signalling pathways, production of ROS, and detoxification of toxin produced by pathogen that were previously described are assigned to part of the plant immune system. Plant immunity is divided in two categories including innate and acquired immune responses. The mechanisms of innate immune response consist of HR, ROSs, cell wall thickening, enzymatic detoxification, and *R* gene-*Avr* gene interaction associated with HR. Acquired immune mechanisms include the accumulation of salicylic acid (SA), systemic acquired resistance (SAR), and induced systemic resistance (ISR)[4,46]. Both innate and acquired immune systems are developed in plants to deal with pathogen infection. In this study, two protein spots (spot no.61 and 63) which corresponded to plant immune resistance were only detected in resistant cultivars. They were characterized as NIM1 and disease resistance protein A19, respectively.

The *NIM1* gene, also known as *NPR1* gene (non-expressor of PR-gene 1), is a crucial master switch gene which relates to the system networks of SAR, ISR, *R* gene-mediated resistance, and the signalling pathway of SA, jasmonic acid and ET[47]. SAR can be operated by the activation of NPR1 and by the presence of SA molecules. After pathogen invasion, accumulation of SA triggers NPR1 activation which then migrates to the nucleus where it binds to the TGA transcription factor, resulting in the stimulation of *PR-1* gene expression[48]. Prior research articles revealed that the overexpression of NPR1 or NIM1 in *Arabidopsis* could enhance the resistance against multiple pathogens[49,50]. Moreover, transgenic rice with the overexpression of the *NH1* gene, *NPR1* homolog, increased the resistance to the bacterial blight pathogen, *Xanthomonas oryzae*[51]. In our study, the expression of *NIM1* in a non-infected resistant cultivar is unexpected. The presence of NIM1 in resistant cultivars may be a key protein marker for further study on selection of disease resistant cultivars in chilli pepper plants.

The disease resistance protein A19 (spot no. 63) is a member of the R protein (plant disease resistance protein) family and serves as the innate immune system in plants. The major components of R protein include a series of leucine-rich repeats (LRRs), a nucleotide binding site and a variable amino-terminal domain. R protein can recognize the pathogen molecules via pathogen associated molecular patterns (PAMP) receptors which subsequently lead to the induction of basal defence responses[52,53]. A previous study demonstrated that the LRR domain of the R protein in *Arabidopsis* recognized the PAMP receptors triggering the MAPK cascade activation following the defence gene expression[4]. In addition, *CALRR1* gene (*R* gene) induced by different pathogens inoculation in pepper plants possibly protected pepper phloem cells from pathogen attack[54]. For these reasons, disease resistance protein A19 may be a pathogen defensive protein in resistant cultivars. As with NIM1, the disease resistance protein A19 may also be used as a molecular marker for selection of resistant cultivars in chilli plants.

This work attempts to study the changes of protein profile in resistant (Mae Ping 80) and susceptible (Long Chilli 455) chilli cultivars upon *F. oxysporum* infection using 2DE and MS techniques. The differential expression of identified proteins correlated with the resistant ability of the two chilli cultivars against fungal infection may provide preliminary clues to understanding the interaction of chilli plants and *F. oxysporum*. To identify the protein spots of interest, PMF data is not enough. However, at least some proteins classified as proteins in solanaceae plants and disease resistance protein A19 in *Capsicum annuum* have been reported. For further study, MS/MS or de novo sequencing is needed.

In conclusion, the defensive proteins against *F. oxysporum* in chilli plants were investigated using a proteomic approach. The comparison of protein patterns between healthy and infected chilli plants revealed that susceptible cultivars showed higher protein responses than resistant cultivars. This result suggests that resistant cultivars have more potential defensive proteins than susceptible ones. Most of the identified responsive proteins were involved in plant defence mechanisms. Protein patterns of healthy resistant and susceptible cultivars were also compared in order to examine the pathogen protective proteins. Two interested proteins identified as NIM1 and disease resistance protein A19 were expressed only in the resistant cultivars. These proteins may serve as biomarkers for screening the *Fusarium* wilt disease resistant chilli cultivars. However, this study represents the first step to understand the chilli plant-*F. oxysporum* pathogen interaction at the proteome level which then could lead to further study on (1) investigation of new chilli varieties expressing high levels of resistance against the *Fusarium* wilt disease and (2) improving plant productivity using selected genes corresponding to pathogen defence.

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