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Analysis of protein profile of tomato root infected with *Fusarium oxysporum* f. sp *lycopersici*

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ABSTRACT. Fusarium wilt caused by *Fusarium oxysporum* f. sp *lycopersici* (Fol) is one of the main diseases affecting tomatoes. The BHRS 2,3 genotype of tomato is, however, resistant to this disease. A proteomic approach was used to understand the defense mechanisms of this genotype using the tomato root, the first tissue that interacts with the fungus, as a target. Protein was extracted and separated by two-dimensional electrophoresis followed by staining with Coomassie brilliant blue. The proteins were identified by MALDI-TOF/TOF mass spectrometry. A total of 22 proteins were identified, 21 of which showed differential expression with 12 proteins being upregulated and nine being downregulated. Plants responded to the pathogen with increased expression of pathogenesis-related proteins. We noted the

induction of proteins involved in hypersensitivity reaction and other defense mechanisms. The expression of proteins of primary metabolism related to energy production, however, decreased, as did the expression of two proteins related to defense against abiotic stress. These results demonstrate the presence of important mechanisms for defense against Fol in the tomato genotype BHRS 2,3.

Key words: *Solanum lycopersicum*; Biotic stress; Fusarium wilt; Proteomics

INTRODUCTION

The fusarium wilt, caused by the soil fungus *Fusarium oxysporum* f. sp *lycopersici* (Fol), is one of the most aggressive diseases affecting the tomato crop. The disease causes great damage, especially in sensitive cultivars and in environments where the air and soil temperature are high, as in tropical countries during the warm seasons (Agrios, 2005). The classical strategies, such as the use of fungicides and crop rotation have not been efficient due to the resistant spores that remain viable for several years, and because of the impact of pesticide residues on human health. Selective breeding has contributed to the emergence of more aggressive new breeds of pathogen. Therefore, it is necessary to develop new and efficient control strategies that do not compromise the environmental safety (Mandal et al., 2009). Resistant cultivars developed by gene transfer appear to be one of the most promising options to combat the disease (Dean et al., 2012). However, resistance to diseases involves a complex mechanism involving recognition of virulence determinants by plant receptors followed by specific signal transduction pathways leading to the production and release of reactive oxygen species, phytoalexins, pathogenesis-related (PR) proteins, in addition to programmed cell death (Torres et al., 2006). Studies on inductors show that resistance to Fol may be related to salicylic acid (Mandal et al., 2009). However, little is known about the molecular relationships among the metabolic pathways, such as recognition, signaling, and plant response, in addition to components not directly related to infection.

Root is the first site to detect and fight soil fungus infections such as Fol. Consequently, understanding the molecular mechanisms happening in this tissue is necessary to improve resistance to the pathogen. Differential proteomics, essentially finds out proteins with different expression levels among different samples caused by a specific factor, is an important part of global proteomic studies. An overview of protein expression can be obtained at high resolution by separating proteins by dimensional (2-DE) electrophoresis combined with peptide identification via tandem mass spectrometry (MS/MS; Manaa et al., 2011; Que et al., 2011). This proteomic approach has become a powerful tool for studying plant development in general and its use has significantly increased in the studies of various stresses, both biotic and abiotic in origin (Faurobert et al., 2007).

The present study analyzed the protein profile of the BHRS 2,3 tomato cultivar, known for its resistance to Fol, with an aim of identifying those with differential expression, using the 2-DE proteomic technologies associated with matrix assisted laser desorption ionization, tandem time of flight mass spectrometer (MALDI-TOF-TOF/MS), which are associated with the defense against the fungus.

MATERIAL AND METHODS

Tomato line and *F. oxysporum* isolate

The wild-type BHRS 2,3 tomato genotype, resistant to fusarium wilt but having no economic value, was used in the present study. The material was procured from the Vegetable Improvement Program of Instituto Agronômico de Pernambuco, Recife, Pernambuco, Brazil. The race 2 isolate of Fol was obtained from the Mycology Collection of the Department of Phytopathology from Universidade Federal Rural de Pernambuco, Brazil.

Experimental design and protein extraction

Seeds were sown in 128-celled Styrofoam trays filled with sterile substrate Plantmax®. Plants, with first-two pairs of true leaves fully open (about 21 days after planting), were removed from the cells with a gentle jet of water to preserve their root integrity. The apical sector (about 2 cm) of the root was removed and then dipped for 20 min in a spore suspension containing approximately 2 x 10^6 conidia/mL, cultured in potato dextrose broth for seven days at 28°C, under continuous light. A group of plants was mock-inoculated with water and used as control. After inoculation, the plantlets were transplanted to 1.5 kg plastic pots containing sterile soil and maintained in a greenhouse. The experimental plots contained three pots, with four plants each, in a randomized block design. Plants were collected 1, 2, 4, and 6 days after the inoculation and were stored at -80°C.

Roots (1000 mg) were ground in ice-cold extraction buffer containing 8 M urea, 4% CHAPS, 10 mM phenylmethylsulfonylfluoride, and 7 mM dithiothreitol (DTT). The mixture was centrifuged at 12,000 *g* for 20 min, and the supernatant collected. Proteins were precipitated with five-volumes ice-cold trichloroacetic acid-acetone (10% trichloroacetic acid in 100% acetone) at -20°C for 6 h and then collected by centrifugation at 20,000 *g* for 20 min. The precipitated protein was centrifuged at 20,000 *g* for 20 min, washed three times in 10 mL ice-cold acetone, with vigorous disruption of the pellet with a glass rod between each wash, and air-dried. The proteins obtained were dissolved in a lysis buffer (8 M urea, 4% CHAPS, 7 mM DTT, and 2% pharmalyte 4-7) at room temperature and stored in aliquots at -80°C, until they were used.

2-DE and image analysis

Protein samples (600 µg) mixed with 250 µL rehydration buffer (containing 8 M urea, 2% w/v CHAPS, 20 mM DTT, and 0.5% IPG buffer; GE Healthcare, Piscataway, NJ, USA), and 0.002% bromophenol blue were loaded onto IPG strips (13 cm), with a linear pH range 4-7 (GE Healthcare, Piscataway, NJ, USA), after a brief sonication and centrifugation. Isoelectric focusing was performed in an Ettan™ IPGphor™ 3 isoelectric focusing system (GE Healthcare, Uppsala, Sweden) following the manufacturer protocol. Before electrophoresing in the second dimension, the IPG strips were equilibrated for 15 min, first in a buffer containing 6 M urea, 30% (w/v) glycerol, 2% (w/v) SDS, and 100 mM Tris-HCl, pH 8.8, supplemented with 100 mM DTT and subsequently for 15 min in the same buffer with 0.25 M iodoacetamide added. The equilibrated IPG strips were transferred onto 12.5% SDS polyacrylamide gels and electrophoresed on an Ettan SE 600 Ruby electrophoresis unit (GE Healthcare, Uppsala,

Sweden). The separated proteins were visualized by staining the gels with Coomassie brilliant blue (CBB)-R250 (Candiano et al., 2004). Images of the CBB-stained gels were acquired at 300-dpi resolution and 16-bit pixel depth, and subsequently analyzed with the ImageMaster 2D Platinum 6.0 (GE Healthcare, Piscataway, NJ, USA) software according to the protocols provided by the manufacturer. The intensity of each spot was normalized by total valid spot intensity. Protein spots were considered differentially expressed if the intensity changed more than two times in the different stages.

MALDI-TOF-TOF/MS

In-gel digestion of proteins was performed as described by Shevchenko et al. (2006), with minor modifications. Trypsin was used at a concentration 25 ng/μL and the in-gel reduction and alkylation steps were omitted. The peptides, thus generated, were dissolved in 10 μL 0.1% trifluoracetic acid (TFA). A saturated solution of alpha-cyano-4-hydroxycinnamic acid (CHCA; 4 mg/mL) in 50% acetonitrile and 0.3% TFA was mixed with equal amounts of sample, spotted on an Anchor Chip 800/384 target plate (Bruker Daltonics, Germany), and dried in laminar airflow for recrystallization. For MS calibration, 0.5 μL peptide calibration standard (Bruker Daltonics, Germany) was spotted on the target with 0.8 μL CHCA matrix and recrystallized, as described above. The samples were analyzed on a MALDI TOF-TOF/MS Ultraflex III (Bruker Daltonics, Germany) used in the reflectron mode. The peptides with a signal-to-noise ratio above 100 were further analyzed by MS/MS using the LIFT technology embedded in the Ultraflex MS; on an average, ten MS/MS spectra were measured for each protein digest leading to the identification of 2-10 peptides. Data processing was realized using the Flex analysis and BioTools software packages (Bruker Daltonics, Germany).

Data analysis was performed using the BioTools 3.0 software, using MASCOT (Matrix Science, London, UK) as a search engine. The parameters were set as follows: database was NCBI (nr); retrieval genera were set as all; data acquisition method was set as combined; maximum missing cut site allowed was set as 1; enzyme was set as trypsin. The quality error scope was set as follows: PMF 0.3D; MS/MS 0.4D.

RESULTS AND DISCUSSION

The time-points for sample collection were chosen based on previous experiments (Malafaia et al., 2013). About 1400-1800 μg protein was extracted from 1 g root tissue of the treated plants. The resolution of approximately 600 μg protein loaded on each gel showed an average of 450-550 spots with pI ranging between 4 and 7 and the molecular mass ranging between 90 and 10 kDa (Figure 1). The 2-DE comparative analysis carried out among the treatments achieved 65% (22 of 34 spots) successful identifications of the proteins. The changes in protein expression, in the plants before and after the pathogen inoculation, were evaluated by comparing the protein profiles. The assessment performed in triplicate revealed 21 differentially expressed spots (Figure 2). Of the identified proteins, the expression of 11 was increased whereas that of 10 proteins was decreased. However, calreticulin, which was present at a high concentration, did not show a differential expression (Table 1). Among the upregulated proteins, two were of unknown function, while among those with known functions, one was involved in primary metabolism, seven in defense against stresses, and one in signaling. Among the identified proteins showing a decreased expression, five were related

to primary metabolism, one to abiotic stress, one to recovery from oxidative stress, and two were of unknown function. The proteins with no differential expression were associated with both primary metabolism and defense.

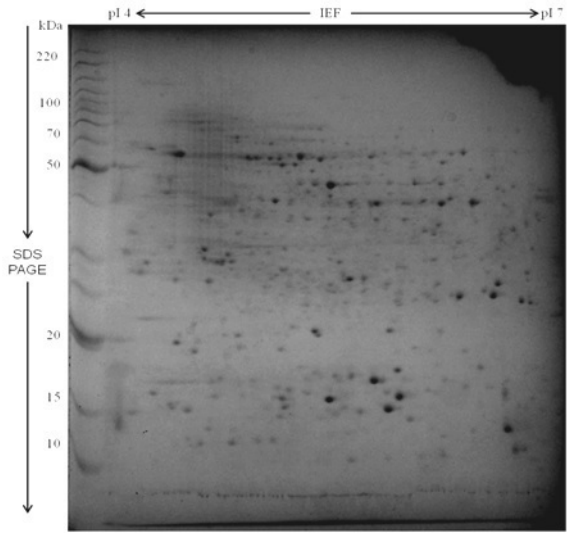

Figure 1. Overview of the proteome of tomato roots infected with *Fusarium oxysporum* f. sp *lycopersici*, separated by two-dimensional electrophoresis. The proteome was resolved through IEF/SDS-PAGE (12.5%) using a linear pI range 4-7 and molecular mass range 10-220 kDa.

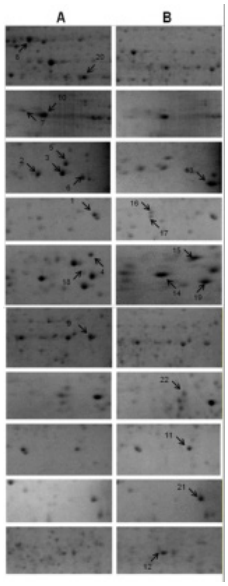

Figure 2. Expanded regions of the 2-D gels showing spots of proteins that were differentially expressed. Panel A shows the spots in the samples from the healthy plant. Panel B shows the spots in the samples from plants inoculated with *Fusarium oxysporum*. The spot numbers indicated by arrows are according to the numbers used in Table 1.

Table 1. List of root proteins that were differentially regulated in the BHRS 2,3 tomato genotype after challenge with *Fusarium oxysporum*.

Spot[a]	Identity[b]	Organism[c]	Accession[d]	Score[e]	pI[f]	Mr[f]	Regulation[g]
1	ATP synthase D chain, mitochondrial, putative	*Solanum demissum*	gi|48209968	48	6.63	19.37	Down
2	Glutathione *S*-transferase, class-phi	*Solanum commersonii*	gi|2290782	58	5.81	23.8	Down
3	Glutathione *S*-transferase, class-phi	*Solanum commersonii*	gi|2290782	58	5.81	23.8	Down
4	17.8-kDa class I heat shock protein	*Solanum lycopersicum*	gi|232273	154	5.84	17.75	Down
5	Triose phosphate isomerase cytosolic isoform	*Solanum chacoense*	gi|38112662	93	5.73	27.25	Down
6	Putative protein	*Arabidopsis thaliana*	gi|3269288	97	6.3	22.35	Down
7	Uncharacterized protein At5g39570	*Vitis vinifera*	gi|225452887	67	4.71	43.01	Down
8	ATP synthase subunit beta, mitochondrial; Flags: Precursor	*Nicotiana plumbaginifolia*	gi|114421	62	5.95	59.93	Down
9	Mitochondrial malate dehydrogenase	*Solanum lycopersicum*	gi|350536645	83	8.87	36.35	Down
10	Calreticulin	*Arabidopsis thaliana*	gi|1009712	71	4.37	46.7	-
11	Predicted protein	*Micromonas* sp *RCC299*	gi|255084065	74	9.26	33.04	Up
12	Endochitinase	*Solanum chilense*	gi|767827	95	6.21	27.53	Up
13	Pathogenesis-related protein (PR-5 protein)	*Solanum lycopersicum*	gi|7414370	43	5.76	27.52	Up
14	RNA-binding glycine-rich protein-1a	*Nicotiana sylvestris*	gi|469070	58	5.58	15.6	Up
15	Glycine-rich protein	*Solanum lycopersicum*	gi|19322	146	9.98	7.3	Up
16	Lemir	*Solanum lycopersicum*	gi|2654440	77	4.83	23.27	Up
17	Lemir	*Solanum lycopersicum*	gi|2654440	77	4.84	23.28	Up
18	40S ribosomal protein S12	*Hordeum vulgare*	gi|12229949	105	5.35	15.62	Up
19	Superoxide dismutase [Cu-Zn]	*Solanum lycopersicum*	gi|350537277	89	5.83	15.4	Up
20	Unknown	*Glycine max*	gi|255638991	62	7.51	36.38	Up
21	Nucleoside diphosphate kinase	*Solanum lycopersicum*	gi|350535074	135	7.04	15.07	Up
22	Actin	*Solanum tuberosum*	gi|3219759	40.7	5.38	41.9	Up

[a]Number allocated according to the numbers used in Figure 1. [b]Identification of protein annotated by mass spectrometry. [c]Plant species from which protein was annotated. [d]Accession number to the database (nrNCBI). [e]MASCOT score. [f]Theoretical molecular weight and isoelectric point computed from the calculation tool Mr/pI from ExPASy. [g]Note of the regulation type suffered by the protein.

Several strategies are employed by plants to perceive and resist stresses induced by pathogens (Kundu et al., 2011). The genotype of BHRS 2,3 tomato has no relevant agronomic characteristics; however, it is resistant to diseases such as fusarium wilt, unlike other genotypes of economic importance. Therefore, studies on defense mechanisms that confer characteristic resistance to this genotype are important for breeding resistant cultivars. Root is the first tissue to interact with the fusarium pathogen; therefore, it was used in this study to identify proteins involved in the defense against this phytopathogen. The proteome of tomato roots has been discussed in previous reports (Li et al., 2008), but such studies have not analyzed the influence of fungal infection.

There was an increase in the plant defense-related proteins after infection with the pathogen. In addition, there was an increased expression of chitinase (PR-3), PR-5 protein, LeMir (*Lycopersicon esculentum miraculin*), nucleoside diphosphate kinase (NDPK), RNA binding glycine rich protein (GRP), superoxide dismutase (Cu-Zn SOD), and actin. Usually, there is an increase in the expression of PR proteins, along with fortification of other responses, when plants are challenged by pathogens. The PR proteins (spots 12 and 13) were present in the unchallenged plants at low concentration, but their expression was observed to increase under pathogen attack or abiotic stress (Dahal et al., 2009). Plant chitinases, which are mostly endochitinases, are reported to participate in the protection, due to their fungal cell wall degrading potential that limits the invasion and growth of pathogen into the host (Ahmed et al., 2012). The PR-5 proteins, also known as thaumatin-like protein due to their similarity with this protein, have been observed to participate in the defense against many pathogens and other abiotic stressors (Ren et al., 2011). Although the mechanisms of action for this protein group have not yet been elucidated, their performance in fighting fungal infections, for example, by inhibition of spore germination and mycelial growth, has been described (Tachi et al., 2009). The induction of PR proteins is considered an activation marker of the basal defense in plants (Dahal et al., 2009).

Hypersensitive reaction (HR) is one of the most efficient forms of plant defense against pathogens (Karrer et al., 1998). LeMir (spots 16 and 17) was secreted and concentrated in roots in response to infestation by nematodes, suggesting its involvement in plant defense against these organisms (Brenner et al., 1998). Moreover, overexpression of this protein was observed in lesions caused by hypersensitive response in tomato plants, demonstrating that LeMir plays an important role in regulating a variety of HR types (Xu et al., 2012). Another HR mechanism is the release of reactive oxygen species (ROS; de Souza et al., 2011). Some ROS, such as H_2O_2, were confirmed as large signaling molecules, promoting greater tolerance of plants to pathogens (Kim et al., 2007). The enzyme SOD (spot 19) is one of the proteins responsible for the antioxidative response, transforming superoxide into hydrogen peroxide (Madanala et al., 2011). The use of SOD to control the ROS concentration in cells is a strategy to activate the signaling of defense pathways such as those involving mitogen-activated protein kinases, which seems to be central in the cellular response to multiple stressors (Apel and Hirt, 2004).

Actin (spot 22) is an essential component in all living beings, responsible for the cytoskeleton formation in several cells of organisms (Franklin-Tong and Gourlay, 2008). In plants, the actin cytoskeleton is also used as a sensor for stress monitoring and is a part of the mechanisms that trigger the HR and programmed cell death (PCD). The actin filaments are reorganized, and accumulate and line up near the infection site, thus allowing isolation of the area where there is PCD (Franklin-Tong and Gourlay, 2008). Although, there is evidence for the actin-ROS interaction during the HR, the fact has not yet been proven (Franklin-Tong and Gourlay, 2008). Thus, increased expression of two LeMir isoforms against the Fol infection as well as that of an enzyme related to ROS and actin, processes related to HR and PDC, is indicative of the resistance of the BHRS 2,3 genotype to this disease.

NDPK (spot 21) is a protein responsible for cellular organization, regulating the nucleotide groups in the cells (Kihara et al., 2011). It has been reported to be involved in signal transduction in plants. In potatoes, NDPK was associated with increased tolerance to multiple stresses (Tang et al., 2008). In rice, there was an increased NDPK1 expression in response to infection by the pathogen, *Xanthomonas oryzae* pv *oryzae*, in addition to elicitors, such as salicylic acid and jasmonic acid, showing a defensive role of NDPK against microorganisms (Cho et al., 2004).

GRP (spots 14 and 15) are involved in various pathways and have a structural function, providing elasticity to the cell wall. They are also involved in post-transcriptional regulation of genes in response to stress (Wang et al., 2012). Increased GRP expression has been reported in response to salt stress, cold, heat, injury, and viral and fungal infections (Kwak et al., 2005). However, their mechanisms of action have not been fully elucidated (Wang et al., 2012). The presence of two isoforms of this protein, with increased expression in response to Fol, corroborates the results of these studies, suggesting that GRP has a role in plant defense.

A decrease in the expression of some proteins associated with primary metabolism (spots 1, 5, 8, and 9) was observed in response to the fungus. ATP synthase is a key protein responsible for energy production (Rexroth et al., 2012). The ROS production, resulting from the infection, directly affects the efficiency of this enzyme due to oxidation of its tryptophan residues, resulting in degradation of the enzyme oxidized by specific proteases (Rexroth et al., 2012). The decreased expression of proteins, such as malate dehydrogenase and triose phosphate isomerase, linked to energy cycles (Hadži-Tašković Šukalović et al., 2011), is related to resource reduction for the fungus and limits its development. Calreticulin is a protein that participates in both the regulation of plant development and protection against abiotic and

biotic stresses (An et al., 2011). With respect to biotic stresses, calreticulins were found to play a role in defense against viruses (Shen et al., 2010). Thus, the presence of this protein in the BHRS 2,3 genotype, even without increased expression, shows its role in different routes, by which it can interact.

However, some proteins related to stress defense showed decreased expression (spots 2, 3, and 4). Glutathione *S*-transferase (GST) is responsible for maintaining the redox state of plants; it combines electrophilic xenobiotics with glutathione, thus reducing their toxicity (Sytykiewicz, 2011). Another defense-related protein that also showed decreased expression upon Fol infection was the class I heat shock resistance (HSP) protein. This group acts as chaperone, linking other proteins, stabilizing unstable structures, and facilitating the modeling of inactive proteins. They are expressed rapidly and abundantly under various stress conditions (Bondino et al., 2012). Decreased expression of HSP and GST proteins during infection is an unusual event. However, the decrease of GST may be related to the fact that during the hypersensitivity reaction there was a need for some ROS being present in the tissue, but this fact needs to be proven.

The present study revealed proteome modifications in the BHRS 2,3 genotype upon the Fol challenge that could contribute to the understanding of this plant-pathogen interaction. The differential expression of the proteins related to defense or stress and primary metabolism in response to the pathogen supports their fundamental role in the plant defense mechanisms. However, further studies are warranted to find other mechanisms and to identify the initiation of processes such as perception of the infection and signaling.

Conflicts of interest

The authors declare no conflict of interest.

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