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Comparative proteomic analysis of the response in resistant and susceptible maize inbred lines to infection by *Curvularia lunata*

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Abstract

Proteins differentially expressed from maize leaves in response to the infection by *Curvularia lunata* strain CX-3 were identified through a high-resolution two-dimensional gel electrophoresis (2-DE) method. Two inbred lines, 78599-1 and E28, were used, respectively, as resistant and susceptible lines to CX-3 infection. Proteins were extracted from the fourth leaves of six- or seven-leaf stage plants sampled at 24, 36, 48, 60, and 72 h after inoculation with CX-3. Twenty-seven differentially expressed protein spots resolved on the 2-DE gels were identified by MALDI-TOF MS/MS. The results showed that these proteins are associated with photosynthesis, respiration, oxidative and drought stress tolerance as well as signal transduction in maize. Among stress-related proteins, the 22 kDa drought-inducible protein, putative glutathione peroxidase (GPX), and translation initiation factor (eIF-5A) were up-regulated in the resistant inbred line and were implicated in host defense response to *C. lunata* infection. It suggests that drought-inducible and oxidation stress-related proteins might directly contribute to maize resistance to *C. lunata*.

Keywords: *Curvularia lunata*; Maize; Proteomics; Two-dimensional electrophoresis (2-DE)

1. Introduction

Maize leaf spot is a disease caused by *Curvularia lunata* (Wakker) Boed and it widely spreads in the world [1]. In 1996, the disease broke out in Northeast China and resulted in a great grain loss of 260 million kilograms [2]. The disease widely spread to more than 10 provinces nationwide over the last two decades [3–5]. Even though great efforts have been made to improve host resistance by germplasm screening and conventional resistance breeding, progress was still slow due to lack of high resistance genes. Additionally, since most genetic markers for resistance are usually selected at the DNA level [6], it has limited progress in selecting molecular markers for resistance breeding due to gene silence or unexpressed mRNA.

Applications of two-dimensional gel electrophoresis (2-DE) and mass spectrometry (MS) have made it possible to overcome the inconsistency between actual levels of resistance and genetic information represented by traditional genetic markers. These techniques allow searching of protein markers with comprehensive genetic information directly related to host resistance, which helps to screen resistant progeny effectively. Moreover, the complexity of disease resistance mechanisms in plants has never been illustrated well relying solely on DNA-based approaches. Studies of proteins and their interactions may provide overall information on defense mechanisms of the host against pathogen infection as compared with DNA-based methods [6].

Many kinds of proteins are involved in the regulation of maize defense responses to pathogen infection [7–12]. For

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instance, the proteins involved in protein synthesis, folding, and stabilization, along with proteins involved in oxidative stress tolerance, are induced in maize embryos when infected by *Fusarium verticillioides*, an ear rot pathogen [8]. In addition, the proteins related to drought or desiccation, water- or osmo-stress, heat-stress, reactive oxygen species (ROS) as well as mitogen-activated protein kinases can be detected by 2-DE [9]. These proteins are considered to be associated with resistance to *Aspergillus flavus* in maize [9–11]. However, there is little information on the relation of differentially expressed proteins in maize leaves to host resistance in response to fungal diseases. The objectives of this study were to separate proteins from seedling leaves of maize inbred lines widely distributed in China by 2-DE and then to identify differentially expressed proteins associated with host defense response to *C. lunata*.

2. Materials and methods

2.1. Plant materials and fungal inoculation

Two maize inbred lines with different levels of resistance to *C. lunata*, 78599-1 (resistant) and E28 (susceptible) [3,4], were used in this experiment. Potted seedlings were grown in a solar greenhouse (25–30 °C).

CX-3, a highly virulent isolate of *C. lunata*, was used for fungal inoculation. It was cultured on potato dextrose agar (PDA) for 4–5 days at 28 °C in darkness. A conidial suspension (10^6/ml in 2% of sucrose and 0.02% of Tween-20) was sprayed onto the whole plants at six- to seven-leaf stages using an air sprayer. Control plants were sprayed with distilled water containing 2% of sucrose and 0.02% of Tween-20. For each inbred line, the fourth leaves from three treated seedlings were sampled at 24, 36, 48, 60, and 72 h after inoculation. They were frozen immediately in liquid nitrogen and stored at −80 °C until protein extraction for 2-DE analysis.

2.2. Protein extraction

About 3 g of each leaf sample was ground in liquid nitrogen and homogenized in five volumes of cold acetone (−20 °C) containing 10% trichloroacetic acid (TCA) and 0.07% 2-mercaptoethanol. The mixture was placed at −20 °C overnight and then centrifuged at 15,000*g* for 30 min at 4 °C. The pellets were resuspended in cold acetone at −20 °C for 1 h, and then centrifuged at 15,000*g* for 30 min at 4 °C. This resuspension step was repeated three times. The extracted proteins were stored at −80 °C overnight and then lyophilized.

2.3. Two-dimensional gel electrophoresis (2-DE)

The protein samples were dissolved with a sample buffer (9.0 M urea, 2.0 M thiourea, 100 mM dithiothreitol (DTT), 4% CHAPS and 0.5% immobilized, pH 3–10 gradient (IPG) buffer) to a final concentration of 1.6 mg/ml. IPG strips (7 cm, pH 5–8, linear gradient, Bio-Rad, USA) were passively rehydrated with 125 μl sample solution (containing 170 μg protein) for 12 h. Isoelectric focusing (IEF) was carried out with the PROTEAM IEF system (Bio-Rad, USA) at 250 V, 1 h, linear; 500 V, 1 h, linear; 1000 V, 1 h, linear; 4000 V, 1 h, linear; 4000 V, 22,000 h, rapid; 500 V, 12 h, rapid.

After IEF, the strips were equilibrated for 15 min with the buffer (50.0 mM Tris–HCl/pH 8.8, 6 M urea, 2% SDS, and 20% glycerol) containing 60 mM DTT, then for another 15 min with the same buffer but containing 25 mg/ml iodoacetamide (IOA). For the second dimensional separation, the equilibrated strips were loaded onto SDS–polyacrylamide gels (12% acrylamide separating gel). Electrophoresis was performed at a constant current of 10 mA for 0.5 h, and then at 20 mA for the remaining running period (about 2–3 h). The entire experimental procedure was repeated three times with multiple gels run per protein extraction.

2.4. Gel staining and image analysis

Gels were stained with Coomassie Brilliant Blue G250 (CBB-G250) as described by Xu et al. [2]. Gel images were acquired by a VersaDoc™3000 image scanner and analyzed with PD-Quest 7.20 software (Bio-Rad, USA). Protein spots were considered to be differentially expressed if at least a twofold intensity variation was observed between the treated samples at least one time point and the control.

2.5. In-gel tryptic digest

Selected protein spots were manually excised from the gels and transferred to a 96-well microplate. Gel pieces were detained with a solution of 25 mM ammonium bicarbonate in 50% acetonitrile (ACN) for 30 min at 37 °C. Then they were washed twice with deionized water, and then dehydrated in ACN. The samples were digested with 12.5 ng/μl trypsin in a buffer containing 25 mM ammonium bicarbonate for more than 12 h at 37 °C. Peptides were then extracted twice using 0.1% trifluoroacetic acid (TFA) in 50% ACN. The extracts were dried under the protection of N_2.

2.6. MALDI-TOF MS and database search

The dried samples were dissolved by 0.8 μl 50% ACN, 0.1% TFA containing 5 mg/ml α-cyano-4-hydroxycinnamic acid (CHCA) (Sigma, St. Louis, USA) before being spotted on the target plate. Samples were air-dried and analyzed by a 4700 MALDI-TOF/TOF Proteomics Analyzer (Applied Biosystems, USA). The UV laser was operated at a 200 Hz repetition rate with a wavelength of 355 nm. The accelerated voltage was operated at 20 kV. Myoglobin digested by trypsin was used to calibrate the mass instrument using the internal calibration mode. Parent mass peaks with a mass range of 700–3000 Da and min-

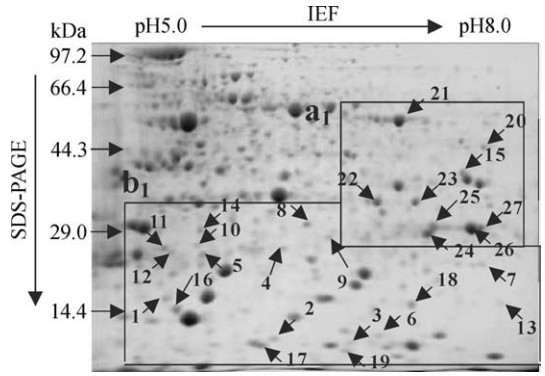

Fig. 1. Profile of the two-dimensional electrophoretic analysis of the proteins extracted from maize leaves (E28, control) with TCA/acetone. There are 27 protein spots (in Boxes a_1 and b_1) differentially expressed between 78599-1 and E28 after inoculation with CX-3 identified by MALDI-TOF MS/MS. The pI range of Box a_1 was from 6.5 to 7.8, and of Box b_1 was from 5.2 to 7.9.

imum *S*/*N* 20 were picked out for tandem TOF/TOF analysis. MS and MS/MS spectra were submitted to MASCOT (V2.1, Matrix Science, UK) by GPS Explore software (V3.6, Applied Biosystems) and searched with the following parameters: NCBInr database, plants, trypsin digest with one missing cleavage, no fixed modification, MS tolerance of 0.2 Da, MS/MS tolerance of 0.6 Da, and possible oxidation of methionine. MASCOT protein scores (based on combined MS and MS/MS spectra) of greater than 64 were considered to be successfully identified.

3. Results

3.1. Proteomic profiles of maize leaves in response to pathogen infection

The total numbers of protein spots on the profile between two inbred lines were not significantly different at 24, 36, 48, 60, and 72 h after inoculation with *C. lunata* strain CX-3 spores. However, differences were detected in the numbers of spots that were up- or down-regulated after inoculation (Table 1). It was clear that at 24 and 36 h after inoculation, there were relatively more down-regulated protein spots than up-regulated protein spots in both

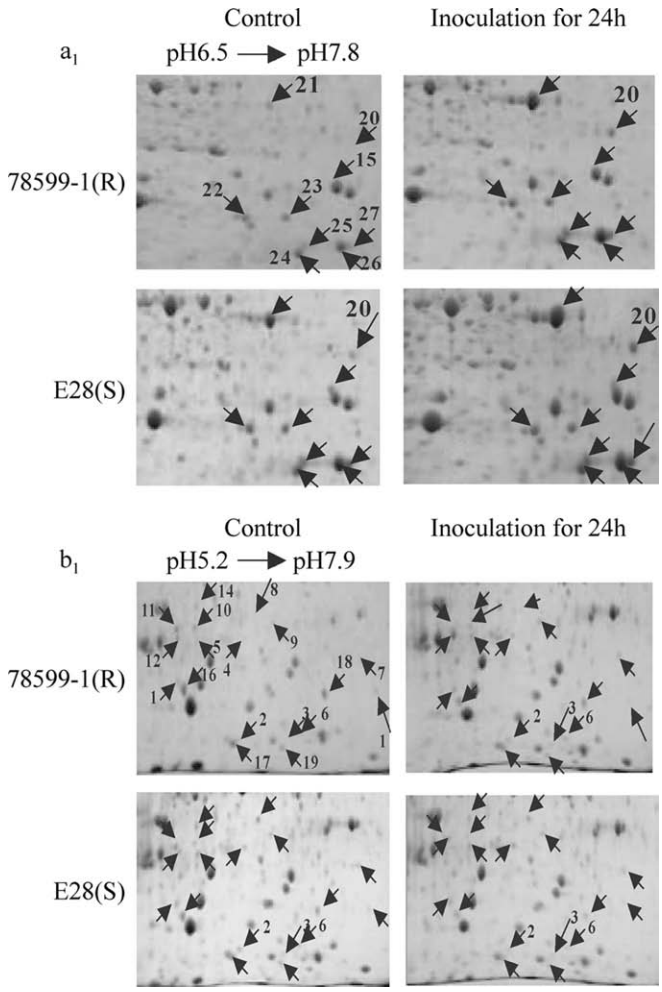

Fig. 2. Details of representative CBB-G250-stained 2-D gels of proteins from leaves of maize inbred lines 78599-1 and E28 inoculated with *C. lunata* strain CX-3 for 24 h. Compared with control, 27 protein spots differentially expressed were pointed out with arrows in the Boxes a_1 and b_1, respectively.

inbred lines. The numbers of up-regulated protein spots gradually increased and eventually became more than down-regulated ones when time increased after inoculation. In general, the numbers of protein spots that displayed quantitative changes after the pathogen infection were higher in 78599-1 than in E28 except at 24 h after

Table 1
Changes in protein spots from 78599-1 and E28 after pathogen infection.

Inoculation times (h)	Total numbers of protein spots*		Numbers of protein spots (twofold)			
	78599-1(R)	E28(S)	78599-1(R)		E28(S)	
			Up-regulated	Down-regulated	Up-regulated	Down-regulated
Control	502 ± 34	513 ± 36	–	–	–	–
24	533 ± 57	518 ± 52	23	28	25	38
36	524 ± 55	521 ± 60	33	38	18	22
48	516 ± 27	496 ± 42	31	34	19	17
60	507 ± 38	516 ± 70	46	31	26	19
72	504 ± 42	512 ± 52	44	32	32	21

* Data represent the mean ± SD of three replicated gels.

inoculation. The difference may indicate that resistant inbred lines may activate more potential defense proteins in response to stimulation of *C. lunata* than susceptible ones. Moreover, some up- or down-regulated proteins were found in both resistant and susceptible inbred lines. These results imply that some common physiological responses to pathogen infection are shared at least partly by resistant and susceptible plants. The results were also verified (data not shown) using another two inbred lines, Luyuan 92 (resistant) and Huangzao 4 (susceptible).

Among the proteins differentially expressed in two inbred lines 78599-1 and E28, 27 protein spots that changed their abundance at least three fold were selected for detailed analysis. These spots were mainly found in two regions of the gels designated as boxes a_1 and b_1 (Figs. 1 and 2 and Table 2). Most of the selected spots were up-regulated in 78599-1 but down-regulated in E28 or up-regulated simultaneously in both inbred lines, especially after 24 h of inoculation. In 78599-1, 14 spots were up-regulated (spots 1, 2, 4–7, 9, 21–27), and 8 spots were down-regulated (spots 10, 11, 13, 14, 16–19) in the inoculated plants. Spots 3 and 20 were newly induced as compared with the control, and spot 12 was up-regulated after 24 h and later down-regulated. In E28, 4 spots increased their expression levels over the course of inoculation (spots 4, 7, 20, 21), but 12 spots decreased in their abundance (spots 1–3, 6, 10–12, 16–19, 25) under the same conditions. Other spots except spots 8 and 15 were up-regulated at an early stage, but changed to be down-regulated at a later time. Spot 8 was found to be constitutively expressed higher in E28 than in 78599-1, and no change in abundance was observed after inoculation. Spot 15 only appeared in E28 and was down-regulated after inoculation.

3.2. Identification of differentially expressed proteins

MS data, including the putative identity, organism, accession number, theoretical pI and Mr value are given in Table 2. Among all the proteins identified, most were found to be closely related to photosynthesis, respiration,

Table 2
Identification of differentially expressed proteins from maize leaves in response to *C. lunata*.

Spot No.	Protein identification	Organism	Accession No.	Mr/ pI	No. of peptides matched	Sequence covered (%)	MASCOT score	Changes after inoculation	
								78599-1	E28
1[a]	Chlorophyll a/b-binding protein precursor (Cab)	*Oryza sativa*	BAD61582	26.2/5.75	9	17.8	441	↑	↓
2[b]	Translation initiation factor 5A (eIF-5A)	*Zea mays*	CAA69225	17.9/5.61	7	54.4	169	↑	↓
3[b]	Putative glutathione peroxidase (GPX)	*Sorghum bicolor*	AAT42166	18.4/6.59	5	22.6	79	+	↓
4[b]	Ascorbate peroxidase (APX)	*Hordeum vulgare*	AAL08495	16.8/5.97	2	20.3	66	↑	↑
5[b]	Cytosolic ascorbate peroxidase (cAPX)	*Z. mays*	CAA84406	27.3/5.28	9	50.0	117	↑	↑↓
6[b]	22 kDa drought-inducible protein	*Saccharum officinarum*	BAB68268	15.9/5.78	6	52.1	96	↑	↓
7[c]	Small ras-related protein	*Nicotiana tabacum*	AAA34109	25.2/6.45	7	31.2	102	↑	↑
8[d]	β-glucosidase aggregating factor precursor (BGAF)	*Z. mays*	AAF71261	31.8/6.08	5	38.9	183	→	→
9[d]	Putative3-β-hydroxysteroid dehydrogenase/isomerase protein (3β-HSD)	*O. sativa*	XP 493881	31.3/9.13	7	17.8	210	↑	↑↓
10[a]	ATP synthase CF1 alpha chain, atpA	*O. sativa*	AAS46118	58.9/6.66	19	41.5	321	↓	↓
11[a]	ATP synthase CF1 alpha chain	*Saccharum* hybrid cultivar	AAT44691	55.7/5.87	25	53.5	312	↓	↓
12[a]	ATPase subunit alpha	*Z. mays*	CAA60283	55.7/5.87	14	30.6	89	↑↓	↓
13[a]	P-pyruvate carboxylase (PPC)	*Z. mays*	CAA33663	109.1/5.77	18	24.9	281	↓	↑↓
14[a]	Fructose 1,6-bisphosphate aldolase precursor (FBA)	*Avena sativa*	AAF74220	41.9/9.01	6	11.3	73	↓	↑↓
15[a]	Glyceraldehyde 3-phosphate dehydrogenase (phosphorylating) (GAPDH)	*Z. mays*	CAA51676	36.5/6.41	8	27.3	71	–	↓
16[a]	Phosphoenolpyruvate carboxylase (PEPCase)	*Z. mays*	ABA33611	20.3/5.28	19	76.9	188	↓	↓
17[a]	PEPCase	*Z. mays*	CAA33317	109.2/5.73	19	26.8	193	↓	↓
18[a]	PEPCase	*Z. mays*	CAA27270	105.6/5.80	24	35.5	384	↓	↓
19–27[a]	Ribulose-1, 5-bisphosphate carboxylase/oxygenase (Rubisco) or Rubisco large subunit							↑	↑↓

[a] Photosynthesis, respiration, and energy metabolism.
[b] Stress-related or inducible proteins.
[c] Signal transduction.
[d] Unknown functions; "+" stands for "induced"; "–" stands for "not observed"; "↑" stands for "up-regulated"; "↓" stands for "down-regulated"; "↑↓" stands for "firstly up-regulated then down-regulated"; "→" stands for "no changes".

responses against stress factors, signal transduction, and protein metabolism. Spots 2, 3, and 6 were best matched to translation initiation factor 5A (eIF-5A), putative glutathione peroxidase (GPX), and 22 kDa drought-inducible protein, respectively. The expression of spot 6 was higher apparently in 78599-1 than in E28 along the time course of infection (Fig. 3).

Differences between the experimental and theoretical values of pI and Mr for some identified proteins were also noticeable (Table 2, Fig. 2). The experimental pI values of spots 6, 7 and 13 were higher than their theoretical values. The experimental Mr values of spots 13 and 17 were considerably smaller than their theoretical ones. Further investigation to illustrate the phenomenon is underway.

3.3. Functional classification of identified proteins in response to C. lunata infection

The identified proteins were classified into four functional groups based on their putative functions (Table 2).

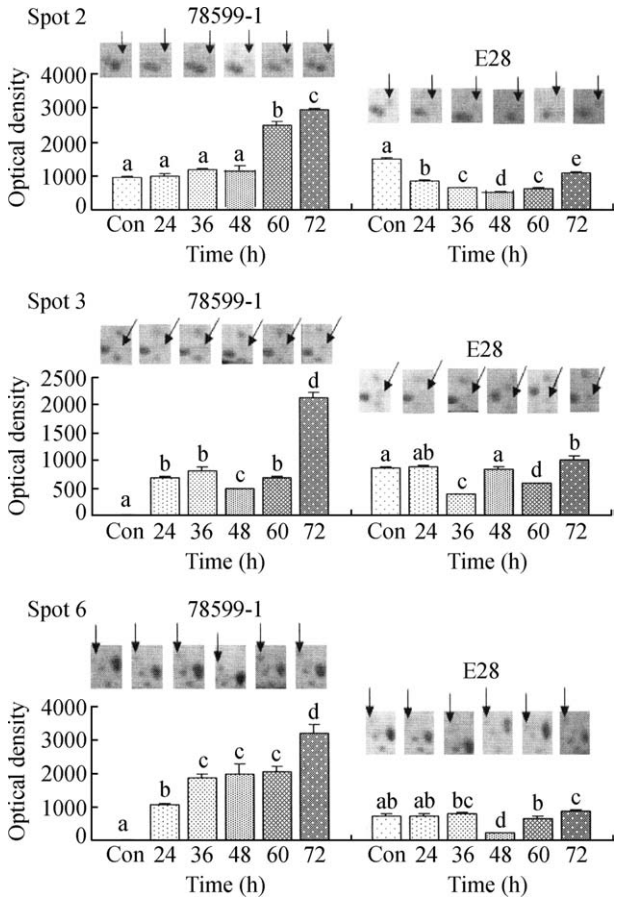

Fig. 3. Different intensity levels of protein spots 2, 3, and 6. Changes in spot intensity are shown by the enlarged gel regions placed over the corresponding relative intensity (histogram). Spot intensity was quantified using PD-QUEST software (Bio-Rad). The relative intensity of a spot was the average of the intensities of the same spot on three replicated gels. Bars indicate ± SD. Different letters above bars indicate a significant difference ($p < 0.05$) according to the LSD test. The spots 2 and 3 were not significantly ($p > 0.05$) different in the expression levels between 78599-1 and E28 while spot 6 was significant ($0.01 < p < 0.05$).

The proteins related to photosynthesis, respiration, and energy metabolism were assigned to Group I, including ribulose-1, 5-bisphosphate carboxylase/oxygenase (Rubisco), chlorophyll a/b-binding protein, phosphoenolpyruvate carboxylase (PEPCase), ATP synthase, ATPase, P-pyruvate carboxylase (PPC), fructose 1,6-bisphosphate aldolase (FBA), and glyceraldehyde 3-phosphate dehydrogenase (phosphorylating) (GAPDH). Group II was composed of GPX, ascorbate peroxidase (APX), 22 kDa drought-inducible protein, and eIF-5A. These proteins were related to stress tolerance and were predicted to be crucial in plant protection against *C. lunata* infection. Group III only contained a small ras-related protein that was involved in signal transduction. Group IV included two proteins, β-glucosidase aggregating factor precursor (BGAF) and putative 3-β hydroxysteroid dehydrogenase/isomerase protein (3β-HSD), their functions in plant stress tolerance are not clear at present.

4. Discussion

The proteomic approach has been successfully used to unravel the resistance mechanisms of maize against kernel rot caused by *Fusarium* or *Aspergillus* [7–10]. We used the 2-DE method to study the responses of maize seedling leaves to infection caused by *C. lunata*. Twenty-seven differentially expressed proteins from inoculated leaves of inbred maize lines were identified by MALDI-TOF/TOF MS and the dynamic changes of these proteins after inoculation were analyzed.

Previous research showed that GPX and APX are involved in the protection of plant tissues against oxidative damage and detoxification of cytotoxic products [8]. Similarly, the correlation between host plant resistance and elimination of active oxygen radicals has been verified [13,14]. In this study we found that stress-related protein GPX could be induced in 78599-1 in response to *C. lunata* infection, and APX was up-regulated in both inbred lines, but the expression was higher in the resistant inbred line. The combined expression of GPX and APX might play a role in supporting maize defense response against *C. lunata* by eliminating excess active oxygen radicals and modulating the balance of H_2O_2.

Certain stress-inducible proteins have been found to contribute to plant defenses in response to pathogens [9,11,15,16]. Drought or desiccation, water-stress related proteins are associated with maize defense response to the kernel rot pathogen *A. flavus* [9]. In this study, a 22 kDa drought-inducible protein was induced by *C. lunata* in resistant inbred line 78599-1, suggesting a correlation between stress tolerance and plant disease resistance [11].

Eukaryotic eIF-5A is one of the factors necessary for the initiation of eukaryotic cellular protein biosynthesis [17]. Even though several eIF-5A DNA sequences have been identified in plants [18,19], the way in which eIF-5A facilitates protein synthesis is not fully understood. In this

study, the expression levels of eIF-5A (spot 2) were increased specifically in the resistant inbred line when infected by *C. lunata*, which suggests that eIF-5A might enhance host defense response by facilitating the translation of mRNA species required for a specific defense response.

In addition to stress-related proteins, the other proteins, particularly those responsible for energy metabolism or signal transduction, may play roles in the induced host plant resistance through a network composed of those proteins. Ras proteins, involved in receptor-mediated signal transduction pathways, can activate mitogen-activated protein kinases (MAPKs), which play important roles in pathogen-induced defense responses [20–22]. All these suggest that stress-related proteins such as GPX and APX, 22 kDa drought inducible protein and eIF-5A are the most likely mediators for resistant performance, or potential molecular markers for the evaluation of maize germplasm resistance against *C. lunata* infection.

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