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Proteomic comparison of four maize inbred lines with different levels of resistance to *Curvularia lunata* (Wakker) Boed infection

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Abstract

Protein profiles of leaves in four maize inbred lines with different disease resistance to pathogen *Curvularia lunata* (Wakker) Boed were studied by two-dimensional electrophoresis (2-DE) and mass spectrometry. Proteins were extracted from the forth leaf of maize seedlings 24 h after fungal inoculation, and fractionated by polyethylene glycol to precipitate the most abundant leaf protein, Rubisco, before gel separation. Protein profiles from 2-DE showed that total numbers of protein spots were increased in all four inbred lines inoculated with *C. lunata* CX-3 strain compared with the control. The numbers of changed protein spots in abundance were higher in resistant inbred lines than in susceptible ones, which implied that resistant inbred lines were more sensitive than susceptible ones to pathogen infection. Among proteins identified by MALDI-TOF MS, germin-like protein GLP and translation initiation factor eIF-5A were supposed to play important roles in maize resistance against *C. lunata* infection.

Keywords: *Curvularia lunata* (Wakker); Maize; Polyethylene glycol fractionation; Proteomics

1. Introduction

Curvularia leaf spot, caused by *Curvularia lunata*, is a major leaf disease after northern and southern leaf blight of corn. The disease occurs severely in hot, humid conditions and can damage the crop significantly. In 1996, the outbreak of the disease caused a great grain loss in Northeastern China [1]. At present, use of resistant varieties is the most efficient way to control the occurrence of this disease. However, evolution of the pathogen to overcome resistance genes of the host often brings great challenges to resistance breeding, so the progress in resistance breeding is urgent to be accelerated, while lack of selectable markers for resistance and poor understanding of host resistance mechanisms have hindered the process greatly [1,2].

Currently, the studies on resistance mechanisms of maize against the pathogen infection remain at a physiological and biochemical level. For example, changes in defensive enzyme activity during the course of pathogen infection have been studied extensively [3,4]. However, genetic pattern of maize resistance to *C. lunata* is found to be based on quantitative inheritance, which implies that many kinds of genes may be involved in the host resistance [5]. Therefore, physiological and biochemical or DNA-based approaches cannot reveal the complexity of disease resistance mechanisms. In our study, a high-resolution two-dimensional gel electrophoresis (2-DE) method combined with mass spectrometry was applied to directly search for protein markers related to host resistance to *C. lunata* in maize and to obtain more information on protein interactions.

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2. Materials and methods

2.1. Plant materials

Four inbred lines with different resistance levels to *C. lunata* were included [6]. They were Luyuan 92 (highly resistant), 78599-1 (resistant), E28 (susceptible), and Huangzao 4 (susceptible). Maize seedlings were grown under natural light in a greenhouse (25–30 °C). At the sixth- or seventh-leaf stage, they were inoculated by *C. lunata*.

2.2. Fungal inoculation

A highly virulent isolate of *C. lunata,* CX-3 [7], was used for fungal inoculation. It was cultured on potato dextrose agar for 4–5 days at 28 °C in darkness. A conidial suspension (10^6/ml in 2% of sucrose and 0.02% Tween 20) was sprayed onto the whole plants with an air sprayer. Plants sprayed with distilled water containing 2% of sucrose and 0.02% Tween 20 were used as control. For each inbred line, the 4th leaves from three treated seedlings were harvested 24 h after inoculation. They were frozen immediately in liquid nitrogen and stored at −80 °C until protein extraction.

2.3. Protein extraction

Rubisco is the most abundant protein (about 50% of soluble protein) in leaves. Presence of Rubisco may hinder high resolution of 2-DE due to the limitation of the loading capacity on isoelectric focusing (IEF) gel, and leads to low abundance proteins difficult to analyze. To remove Rubisco, samples were extracted and fractionated with PEG before being loaded on the IEF gel according to Kim et al. [8]. About 3 g of samples was transferred to a prechilled mortar and ground with a pestle in liquid nitrogen to a fine powder. The powder was homogenized in 18 ml (6 volumes) of ice-cold Mg/NP-40 buffer containing 0.5 M Tris–HCl, pH 8.3, 2% NP-40, 20 mM $MgCl_2$, 2% 2-mercaptoethanol, 1 mM phenyl methyl sulfonyl fluoride, and 1% polyvinyl polypyrrolidone. After centrifugation at 12,000*g* for 15 min at 4 °C, the proteins in the supernatant were subjected to PEG fractionation by adding a 50% PEG stock solution to a final concentration of 20% PEG. The mixed sample was incubated on ice for 30 min and then centrifuged at 12,000*g* for 15 min at 4 °C. Proteins in the supernatant were precipitated by adding four volumes of cold acetone 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.

2.4. Isoelectric focusing (IEF)

The protein samples were resolubilized with a sample buffer to a final concentration of 1.6 mg/ml. The buffer consisted of 9.0 M urea, 2.0 M thiourea, 100 mM DTT, 4% CHAPS and 0.5% immobilized pH gradient (IPG) buffer. IEF was carried out with the PROTEAM IEF system (Bio-Rad, USA) using IPG strips (7 cm, pH 5–8, linear gradient, or 17 cm, pH 5–8, linear gradient, both from Bio-Rad, USA). The strips were passively rehydrated for 12 h with 125 μl sample solution containing 170 μg protein for 7 cm strips, or 350 μl sample solution containing 100 μg protein for 17 cm strips. The voltage settings for 7 cm strips were 250 V, 1 h, linear; 500 V, 1 h, linear; 1000 V, 1 h linear; 4000 V, 1 h linear; 4000 V, 22,000 Vh, rapid; 500 V, 12 h, rapid. The settings for 17 cm IPG strips were 250 V, 0.5 h, linear; 1000 V, 1 h, rapid; 8000 V, 3 h, linear; 8000 V, 60,000 Vh, rapid; 500 V, 12 h, rapid.

2.5. SDS–PAGE

After IEF, the strips were equilibrated for 15 min with a buffer containing 50.0 mM Tris–HCl, pH 8.8, 6 M urea, 2% SDS, 20% glycerol, and 60.0 mM DTT, then for another 15 min with the same buffer but containing 25.0 mg/ml iodoacetamide. For the second dimensional separation, the equilibrated strips were loaded onto SDS polyacrylamide gels (12% acrylamide separating gel, $7 \times 7 \times 0.1$ cm for 7 cm strips or $17 \times 17 \times 0.15$ cm for 17 cm strips), and sealed with 0.5% agarose. For 7 cm gel strips, electrophoresis was carried out at a constant current of 10 mA per gel for 0.5 h, and then at 20 mA per gel for the remaining running period (about 2–3 h). For 17 cm gels, a constant current of 30 mA per gel was applied for the first hour, and then at 50 mA per gel for the remaining running period (about 6–7 h). The entire 2-DE experiment was repeated three times with multiple gels run per protein extraction.

2.6. Gel staining and image analysis

Coomassie brilliant blue G-250 (0.25%) was used to stain 7 cm preparative gels as described by Xu et al. [9]. For 17 cm analytical gels, silver staining was performed as follows: 50% ethanol, 50% acetic acid, 20 min; washed with deionized water twice, each for 30–60 min; 0.02% sodium thiosulfate, 1–2 min; washed with deionized water twice, each for 1 min; 0.1% silver nitrate (precooling at 4 °C), 20–40 min;0.04% formaldehyde in 2% sodium carbonate, 2–3 min.

Gel images were acquired by the VersaDoc^{TM}3000 image scanner and analyzed with PD-Quest 7.20 software (both from Bio-Rad, USA). Protein spots were considered to be differentially expressed if at least a twofold intensity variation was observed between the inoculated samples and the control.

2.7. In-gel tryptic digest

Selected protein spots were manually excised from Coomassie brilliant blue stained preparative gels and transferred

to a 96-well microplate. Gel pieces were destained with a solution of 25 mM ammonium bicarbonate in 50% acetone for 30 min at 37 °C. Then they were washed twice with deionized water, and shrunk in acetone. The samples were then swollen in a digestion buffer containing 25 mM ammonium bicarbonate and 12.5 ng/μl trypsin at 4 °C. After 30 min incubation, the gels were digested for more than 12 h at 37 °C. Peptides were then extracted twice using 0.1% TFA in 50% acetone. The extracts were dried under the protection of N_2.

2.8. Mass spectrometry and database search

The dried samples were dissolved in 0.8 μl matrix solution (5 mg/ml a-Cyano-4-hydroxycinnamic acid in 50% acetonitrile, 0.1% TFA) before being spotted on the target plate. Samples were allowed to air-dry and analyzed by 4700 MALDI-TOF/TOF Proteomics Analyzer (Applied Biosystems, Foster City, CA, USA). The UV laser was operated at a 200 Hz repetition rate with 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 mass range 700–3200 Da and minimum S/N 20 were picked out for tandem TOF/TOF analysis. Combined MS and MS/MS spectra were submitted to MASCOT (V2.1, Matrix Science, London, UK) by GPS Explore software (V3.6, Applied Biosystems) and searched with the following parameters: NCBInr database, plants, trypsin digest with one missing cleavage, none 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 comparison of maize leaves in response to C. lunata infection

Over 700 protein spots were detected from each silver-stained gel after removal of Rubisco by PEG fraction. As compared with control, the total number of protein spots in all four inbred lines was increased 24 h after inoculation of CX-3. The number of protein spots that displayed quantitative changes (at least twofold variation in abundance) was higher in resistant inbred lines than in susceptible ones, and more up-regulated protein spots were detected in resistant ones, especially in Luyuan 92 (Fig. 1, Table 1).

From over 100 differentially expressed protein spots, 10 protein spots were selected for detailed analysis by mass spectrometry. Eight of them (spots 1–8) were located in frames A and B and the other two (spots 9, 10) were in frame C. Spots 1–8 were considered to be tightly related with *Curvularia* resistance based on changes in abundance after inoculation of four inbred lines with different resistance levels. After inoculation of CX-3, spot 1 was specifically induced in resistant inbred lines Luyuan 92 and 78599-1, and the expression levels of spot 2 and spot 3 were increased in Luyuan 92, while no obvious changes were observed in other three inbred lines. As to spot 4, it was up-regulated in both resistant inbred lines and remained unchanged in susceptible ones. Spot 5 increased its abundance remarkably in Luyuan 92 and decreased in the other three inbred lines especially in Huangzao 4. In frame B, the expression levels of spot 6 and spot 8 did not change in Luyuan 92 after inoculation, while they increased in other three inbred lines especially in Huangzao 4. As to spot 7, its abundance distinctly decreased in Luyuan 92, but specifically induced or increased in the other three inbred lines. In frame C, spot 9 and spot 10 were both up-regulated in Luyuan 92 and Huangzao 4, while no change was observed in 78599-1 and E28 (Fig. 2).

3.2. Identification of differentially expressed protein spots

Mass spectrometry data of ten selected protein spots, including the putative identity, organism, and accession number, are given in Table 2. Eight of them were successfully identified. Spot 1 and spot 2 were both germin-like proteins (GLPs). Spot 3 and spot 5 were both the oxygen-evolving enhancer protein 1 (OEE 1). Spot 4 was identified as translation initiation factor 5A (eIF-5A). Spots 8, 9, and 10 were recognized as 5-bisphosphate carboxylase/oxygenase (Rubisco), oxygen-evolving complex (OEC), and as oxygen-evolving enhancer protein 2 (OEE 2), respectively. As to spot 6 and spot 7, they were failed to be identified.

4. Discussion

Compared with DNA- or mRNA-based methods, proteomics has been proved to be more effective to screen disease resistance-related proteins and uncover resistance mechanisms by displaying changes in protein expression patterns [10]. Until now, proteomic approaches have been successfully used in discovering the resistance mechanisms in maize against kernel rot caused by *Fusarium* or *Aspergillus* [11–13], while little has been reported on maize leaf fungal disease. Therefore, a 2-DE system to separate proteins from maize seedling leaves was first established to identify proteins differentially expressed during the process of pathogen infection caused by *C. lunata*. In order to avoid the interference of genetic backgrounds, four inbred lines with different resistance levels were included. Resistant inbred lines with more changed protein spots implied that resistant hosts seemed to be more responsive to pathogen infection.

Among differentially expressed proteins identified, GLP (spot 1 and spot 2) was found to be newly induced or up-regulated in resistant inbred lines after inoculation. It is considered to be associated with host defense response. A previous study suggests that GLPs exist in all organs and developmental stages, and some GLPs are also involved in response to various stress conditions including fungal

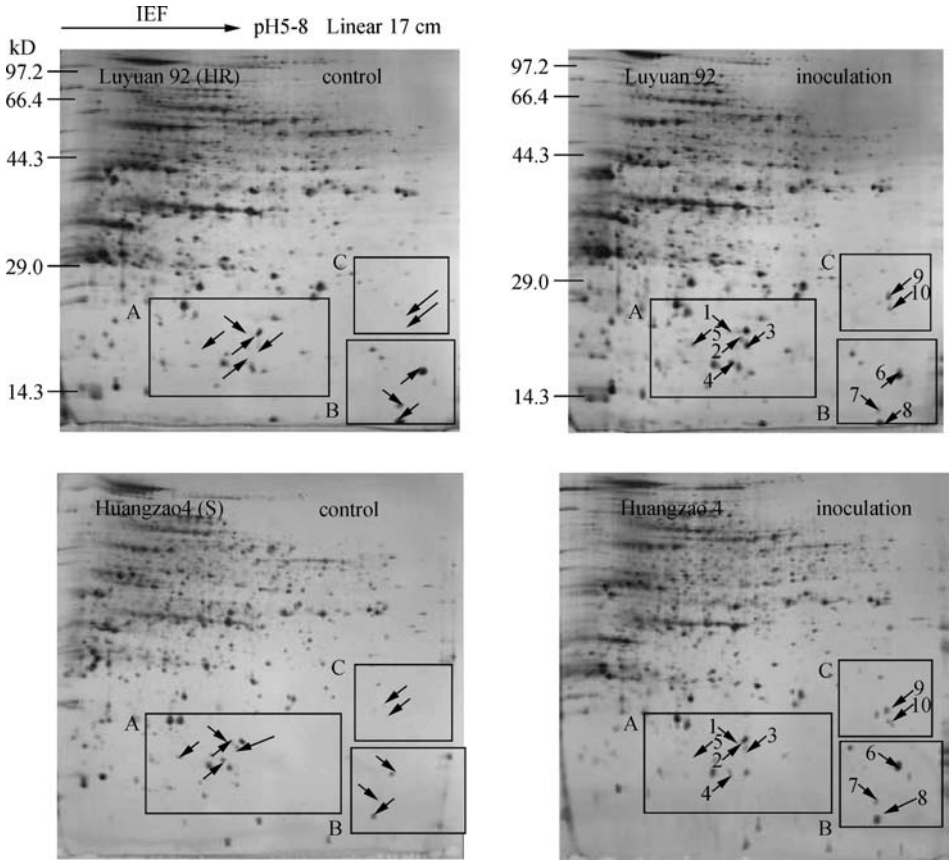

Fig. 1. 2-DE maps of maize leaves in response to *C. lunata* CX-3 infection (silver-stained). "HR" stands for "highly resistant", "S" stands for "susceptible". 1–10 are protein spots.

Table 1
Changes in number of protein spots among inoculated inbred lines with CX-3 for 24 h.

Inbred lines	Total protein spots separated		Numbers of protein spots (two-fold)	
	Before inoculation	After inoculation	Up-regulated	Down-regulated
Luyuan 92 (HR)	771	882	42	33
78599-1 (R)	737	760	27	27
E28 (S)	754	791	26	24
Huangzao 4 (S)	710	768	32	27

attack [14]. A significant increase in GLP activity was detected in barley in response to powdery mildew fungus, *Erysiphe graminis* f.sp. *hordei* [15]. It has been shown that some GLPs possess the enzymatic activity of superoxide dismutase (SOD) or oxalate oxidase (OXOX), which is related to the production of hydrogen peroxide (H_2O_2) [14]. As a signal molecule, H_2O_2 regulates host defense responses during the process of pathogen attack [16–18]. Meanwhile, SOD is a well-known scavenger of active oxygen radicals. Therefore, it could protect host cell membrane from damages caused by active oxygen radicals during pathogen infection. However, there are also some GLPs lacking OXOX or SOD activities but still functioning in host resistance. They may play a structural role in cell-wall reinforcement [16]. In maize, one GLP gene, *Zmglp1,* has been cloned through cDNA-AFLP [19], and its exact roles in maize in response to *C. lunata* infection still need to be clarified.

Eukaryotic translation initiation factor 5A (eIF-5A) (spot 4) is another important protein up-regulated in the resistant inbred lines in response to pathogen infection. It is known that eIF-5A is one of the factors necessary for the initiation of eukaryotic cellular protein biosynthesis, and it is also involved in mRNA selective transport. In tomato, eIF-5A was supposed to facilitate the translation of mRNA required for cell death [20]. We deduce that eIF-5A might assist the translation of mRNA species required for defense response to enhance host resistance to pathogen infection. EIF-5A as well as other stress-related proteins such as 22 kDa drought inducible protein was found to be induced or up-regulated in inoculated resistant inbred lines (data not

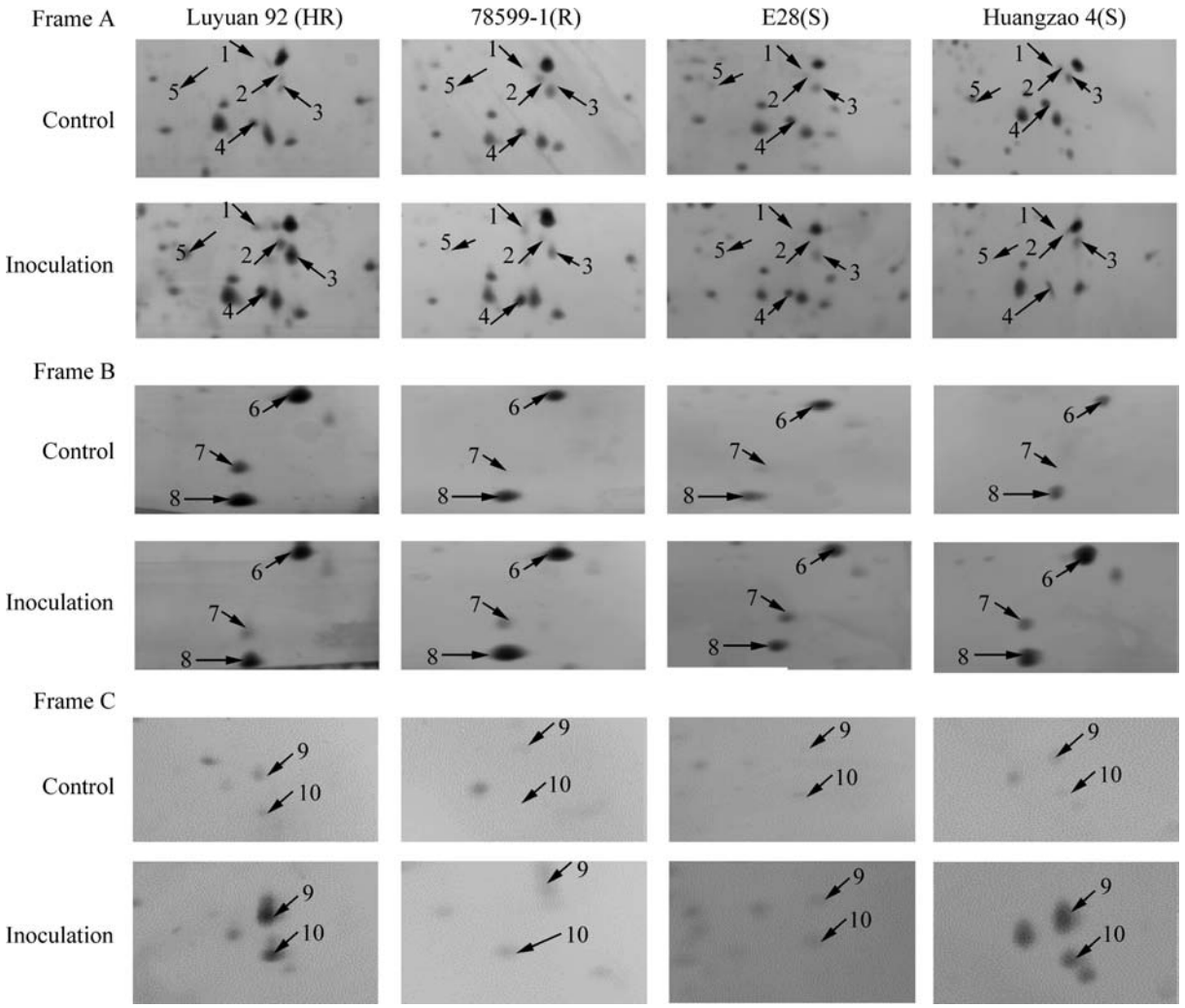

Fig. 2. Detailed comparison of spots 1–10 in inoculated inbred lines with *C. lunata* CX-3. "HR" stands for "highly resistant"; "R" stands for "resistant"; "S" stands for "susceptible".

Table 2
MS results of ten differentially expressed protein spots.

Spot No.	Protein identification	Organism	Accession No.	Trends in expression after inoculation			
				Resistant		Susceptible	
				Luyuan 92	78599-1	E28	Huangzao 4
1	Germin-like protein (GLP)	*Zea mays*	gi|37623879	+*	+*	–	–
2	Germin-like protein (GLP)	*Zea mays*	gi|37623879	↑	↓	↓	↓
3	OEE1	*Fritillaria agrestis*	gi|11133881	↑	→	→	→
4	eIF-5A	*Zea mays*	gi|1546919	↑	↑	→	→
5	OEE1	*Bruguiera gymnorrhiza*	gi|9229957	↑	↓	↓	↓
6	Unsucceed			↓	↑	↑	↑
7	Unsucceed			↓	+	↑	+
8	Rubisco	*Exbucklandia populnea*	gi|3851506	↓	↑	↑	↑
9	23 kDa OEC	*Spinacia oleracea*	gi|21265	↑	→	↑	↑
10	OEE2	*Oryza sativa*	gi|34899580	↑	↑	→	↑

Note: "*" stands for "special"; "+"stands for "induced"; "–"stands for "not observed"; "↑"stands for "up-regulated"; "↓"stands for "down-regulated"; "→"stands for "no changes".

shown), so we postulate that there might be certain cross-talk between stress tolerance and disease resistance in plants [13].

The expression levels of OEE 1, OEE 2, and OEC were also found to be significantly up-regulated in highly resistant Luyuan 92 after inoculation. These proteins are nuclear-

encoded, and peripherally bound to photosystem II (PSII) on the luminal side of the thylakoid membrane [21]. In addition to their energy roles, these proteins may have defense functions. For instance, OEE2 activated by a wall-associated kinase 1(Wak 1) is thought to modulate formation of reactive oxygen species (ROS), which have functions in defense signaling, induction of defense-related proteins and regulation of hypersensitive reaction (HR) [22]. Meanwhile abiotic stresses such as salinity and high light could also change the expression levels of OEE or OEC [21,23]. It was suggested that there might also be some cross-talk between defense pathways and photosynthesis. Defense signaling molecules might induce the expression of proteins related to the photosynthesis.

In the study, with the 2-DE system established, proteins that may be related to host resistance were identified during *C. lunata* infection. Their exact roles in defense response need to be determined by the corresponding gene's function analysis. In this study, 2 out of 10 protein spots were failed be identified, with more information provided by genome and proteome databases, we are sure that more and more important proteins related to disease resistance will be identified, and the resistance mechanisms will be clarified comprehensively.

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