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RESEARCH ARTICLE

Cucumber Response to *Sphaerotheca fuliginea*: Differences in Antioxidant Enzymes Activity and Pathogenesis-Related Gene Expression in Susceptible and Resistant Genotypes

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Abstract: Cucurbits powdery mildew is one of the most detrimental diseases of cucumber plants worldwide. A detailed insight into the biological processes leading to resistance or susceptibility to the pathogen would pave the road for an efficient disease-resistance breeding program. In the present study, the molecular and biochemical responses of a resistant vs. a susceptible cucumber cultivar infected with *Sphaerotheca fuliginea* were investigated. The alterations in the activity of two antioxidant enzymes i.e. superoxide dismutase (SOD) and catalase (CAT) were analyzed during different time courses. The changing pattern of the expression of *PR-8* gene (chitinase class III) was evaluated through qPCR. Results showed that the *PR-8* gene expression was raised in the leaves of both cultivars 96 hours post inoculation (hpi), however, with a 6 times higher expression rate in resistant cultivar compared to the susceptible one. The results imply that *PR-8* may be a key factor of resistance to the pathogen. For both cultivars, SOD showed similar activity pattern and was raised at the early hours post inoculation and showed a peak 6 hours post inoculation with higher activity in the resistant cultivar. In contrast, CAT showed distinct activity patterns between cultivars and showed comparatively higher activity in the susceptible host. The possible reasons for these differences are discussed. The results of the present work give a more clarified insight into the possible mechanisms behind the resistance to cucumber powdery mildew caused by *S. fuliginea*.

Keywords: Cucumber, Chitinase class III, Antioxidant activity; *PR-8* gene

INTRODUCTION

Cucumber (*Cucumis sativus* L.) is one of the widely grown and economically important vegetables throughout the world [1] which is constitutively under risk of attacks by fungal pathogens. Powdery mildew, caused by *Sphaerotheca fuliginea* F. cucumidis Jacz. is a widely distributed disease of cucurbits affecting most cucumber cultures in greenhouse and field conditions [2-4]. In addition, development of greenhouse cultures has provided a favorable condition for the pathogen to maintain over the year and spread much severely. Pathogenesis related (PR) proteins PR-8, known also as chitinase class III and cucumber chitinase are an important group of plant pathogenesis related proteins [5] participating in a broad stress responses of plants. Since chitin is present in the cell wall of fungi, the study of chitinases is quite relevant when analyzing the fungal attack. Induction of chitinase expression by fungal, bacterial or viral infections is well documented [6]. In a study on melon plants, a strong relationship between resistance to *Fusarium oxysporum* and chitinase class III

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have been reported [7]. In addition to cucurbits, chitinase class III showing both endo- and exochitinase activity have been isolated in the leaves of other plants [8]. In melon plants heat shock protein has been reported to induce expression of Chitinase1 enzyme along with salicylic acid resulting in systemically increasing resistance to gray mold fungus [9]. Depending on the type of plant resistance mechanism, after pathogen attacks, accumulation of reactive oxygen species (ROS), particularly superoxide free radicals (O^{2-}) and hydrogen peroxide (H_2O_2), increases rapidly to inhibit further development of pathogens. This is while there are several enzymes eliminating ROS in infected plants. Conversion of the superoxide free radicals (O^{2-}) to molecular oxygen and H_2O_2 is catalyzed by superoxide dismutase (SOD). Elimination of H_2O_2 is catalyzed by catalase (CAT), peroxidases and other scavenging enzymes [10]. There are a number of reports addressing antioxidant enzyme induction associated with the defense against plant pathogens fungi [11,12]. While, in other reports, particularly in relation to biotrophic fungal pathogens, plant defense responses have been associated to inhibition of antioxidant activity and accumulation of H_2O_2 [13], where, H_2O_2 participation in defense mechanisms e.g. direct antimicrobial activity, papilla formation [13], inhibition of fungal spore germination [14], hyper-sensitive response (HR) [15] and inducing signaling pathways of systemic acquired resistance (SAR) [16] has been reported.

To our knowledge, despite many investigations on plant pathogen interactions, there are just few reports [17-20] of studies comparing the responses of a resistant vs. a susceptible plant genotype. The objectives of the present work were to comparatively study the response of a resistant vs. a susceptible cucumber cultivar infected with *S. fuliginea* aiming to document possible differential responses of the cultivars.

MATERIALS AND METHODS

Plant material

Powdery mildew resistant Green Magic and susceptible Super N3 cucumber (*Cucumis sativus* L.) cultivars were chosen among a collection of cucumber cultivars we recently investigated and were screened in another study to identify resistant and susceptible cultivars [21]. A number of 50 seeds per cultivar were grown in 15 cm-diameter plastic pots and randomly arranged on greenhouse benches. Growing was conducted under controlled conditions with a 14/10 h light/dark photoperiod, average temperatures of 24ºC for days and 18ºC for nights and a range of 30-50% relative humidity. The soil composition was 1:1:1 perlite, peat moss and coco peat. Once every four days, plants were watered to saturation with 0.1% NPK (20:20:20) fertilizer solution.

Pathogen culture and plant inoculation

The pathogen was isolated from infected greenhouse cultures and maintained on susceptible plants under controlled conditions. *S. fuliginea* inoculum suspension was prepared from freshly sporulating leaves by immersing a few pieces of leaves in 200 ml tap water containing 200 µl Tween 20 as surfactant and adjusted to $3\text{-}5\times10^4$ conidia/ml. For pathogen inoculation, plants with first fully expanded true leaf, were transferred to an inoculation room with a suitable temperature and relative humidity. The upper surface of the leaves was inoculated by spraying uniformly with a hand sprayer until tiny water droplets covered the leaf surface but not flawed. After 30 minutes, as the surface of the leaves dried out, plants were kept in darkness for 24 hours with relative humidity and temperature ranges of 70-80% and 15-20°C, respectively. The room conditions were then set on a night/day relative humidity ranges of 75% / 60% and temperature ranges of 18°C / 24°C, respectively. Leaf samples were harvested in three biological replicates over the time courses of 0, 6, 12, 24, 48, 72 and 96 Hours Post Inoculation (hpi) and then immediately transferred to liquid nitrogen and kept in -80°C. All experiments including gene expression analysis and biochemical assays were performed for three biological replicates.

Analysis of *PR-8* expression using Quantitative RT-PCR (qPCR)

Total RNA from leaf samples was extracted using an optimized protocol using TRIzol reagent (Invitrogen) following manufacturer recommendations. All RNA samples were treated with DNase I (Thermo Scientific) for 15 min at 37ºC for elimination of possible genomic DNA contaminants. cDNA was synthesized using 2 µg of total RNA, oligo(dT) primers, RevrtAid reverse transcriptase and RiboLock RNase Inhibitor (Thermo Scientific) according to the standard protocol suggested by manufacturer. Final cDNA reactions were diluted 5 times with TE buffer. Before the main qPCR an PCR was

done for 30 cycles (94°C for 30 s, 60°C for 30 s, 72°C for 1 min) using 2 µl cDNA as template. The PCR products were separated on 2% agarose gel and visualized under UV-transilluminator after staining with ethidium bromide.

Primers for qPCR were picked using Primer3 online software and were checked again using OLIGO5 analyzer software. All assays were validated using standard curve validation procedures. PCR was carried out using the one step SYBR® Green RT-qPCR with hot-start *Taq* DNA polymerase (Thermo Scientific) in a BIO-RAD real-time PCR machine (CFX96™ Touch Real-Time PCR Detection System) following manufacturer's recommendations and with a 60°C annealing temperature and 40 cycles. A housekeeping gene (*Actin*) was used as reference gene for normalization, and comparative gene expression method ($2^{-\Delta\Delta cT}$) was used for data analysis. Genes transcript accession numbers, amplicon sizes and the primers are given in Table 1. Relative quantification of gene expression was determined by amplifying the target *PR-8* and the reference actin genes. The specificity of the PCR reactions was confirmed by melting curve analysis of the products, as well as by size verification of the amplicons on 2% agarose gel.

Total protein content

For total soluble protein content, 200 mg of leaf tissues was powdered in liquid nitrogen using a pre-chilled pestle and mortar and then homogenized in one ml homogenization potassium phosphate buffer (100 mM potassium phosphate buffer at pH 7.0 containing 0.5 mM EDTA). The homogenate was then centrifuged at 17000 g for 20 minutes at 4°C and the supernatant used for determination of the total protein content and enzyme activities. The total soluble protein concentration was measured according to the Bradford [22] method and bovine serum albumin (BSA) was used as standard.

Table 1. Genes and related primers used for qPCR.

Gene Name (Accession No.)	Primer sequences (5' to 3')
PR-8 (M24365.1)	GCGGTTTTGGATGGCGTTGAT
	GTCTAGGTGAGCGTCTGGTA
Actin (AB010922.1)	GATTCTGGTGATGGTGTGAGTC
	TCGGCAGTGGTGGTGAACAT

SOD activity assay

The activity of SOD was determined according to a previously described method [20] with some modifications. Three tubes with the same reaction conditions were used including: 1.5 ml of 50 mM phosphate buffer, 0.3 ml of 130 mM Met, 0.3 ml of 750 mM nitro blue tetrazolium (NBT), 0.3 ml of 100 mM EDTA-Na_2 and 0.5 ml of water. The enzyme extract (0.05 ml) was added to the first tube and then exposed to 4000 lux light for 15 min at 25°C. The second tube was also exposed to the above mentioned light conditions, while the third tube was stored in the dark. One unit of SOD activity was defined as the amount of enzyme required to cause a 50% inhibition in NBT reduction at 560 nm wavelength.

CAT activity assay

CAT activity was determined using the method described by Aebi [23] with slight modification, wherein CAT activity was estimated based on declining of absorbance of oxygen peroxide H_2O_2 at 240 nm. A 2 ml reaction mixture was used containing 30 mM H_2O_2, 50mM phosphate buffer (pH 7) and 0.05 ml protein extract.

Data analysis

Relative expression levels of mRNA for *PR-8* gene were analyzed using BIO-RAD CFX manager software (version1.1.308.11111), and the transcript abundance of chitinase expression was normalized based on that of actin as reference gene. Data obtained from enzyme assays analyzed using the SAS 9.1 statistical software.

RESULTS

Analysis of *PR-8* gene expression

Comparison of *PR-8* expression profiles in the resistant and susceptible cucumber cultivars showed significant differences in gene expression rates (Figure 1). For both cultivars, in the first 12 hpi there was no significant increase in chitinase expression compared with the control (0 hpi). A slight and negligible increase in the gene expression was observed from 24 to 48 hpi. This is while the two cultivars showed a significant rise in the gene expression at 96 hpi, but the gene expression rate of the resistant cultivar was dramatically higher than that of the susceptible one, so that, the expression of resistant cultivar raised by 6 times (more than 300 normalized relative expression) higher than that of susceptible

cultivar Super N3 (less than 50 normalized relative expression) (Figure 1).

SOD activity

Compared to the control (0 hpi), activity of SOD showed a significant alteration during the time course after inoculation in both resistant Green Magic and susceptible Super N3 cultivars. The pattern of SOD activity alteration was almost the same for both cultivars so that the enzyme activity was raised during early hours of post infection time, was declined after 6 hpi, reached near its initial rate at 24 hpi and then remained constant until the end point of the experiment i.e. 96 hpi (Figure 2).

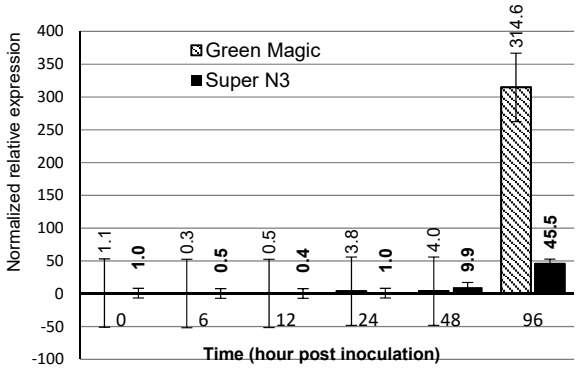

Figure 1. Relative gene expression profile of *PR-8* over a time course from 0 to 96 h in resistant (Green Magic) and susceptible (Super N3) cucumber cultivars after inoculation with powdery mildew caused by *S. fuliginea*.

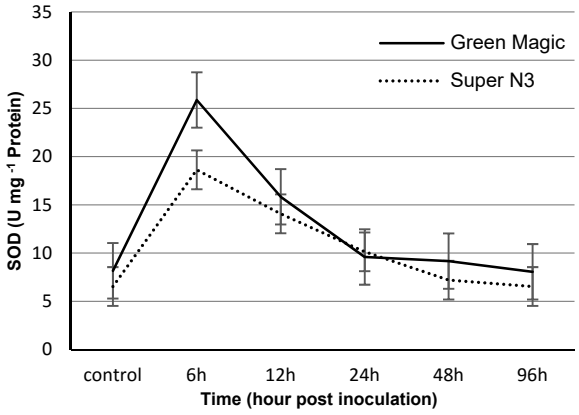

Figure 2. Changes in the activity of superoxide dismutase (SOD) enzyme over a time course from 0 to 96 h post inoculation with *S. fuliginea* in the resistant (Green Magic) and the susceptible (SuperN3) cucumber cultivars.

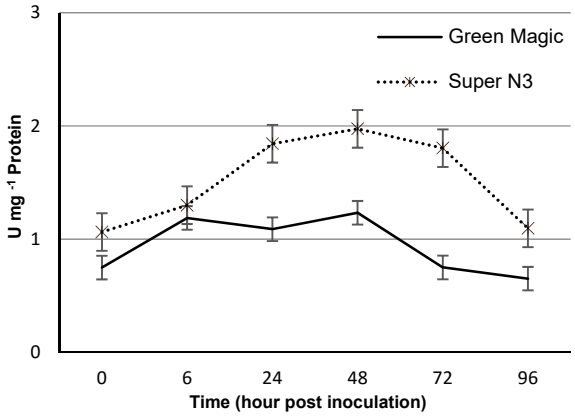

Figure 3. Alterations of the activity of catalase (CAT) enzymes over a time course from 0 to 96 h post inoculation of *S. fuliginea* in the resistant (Green Magic) and susceptible (SuperN3) cucumber cultivars.

CAT activity

CAT activity significantly differed over time in both resistant and susceptible cultivars, however, in contrast to SOD, the pattern of CAT activity was dramatically different between two cultivars (Figure 3). In resistant cultivar (Green Magic), CAT activity was significantly raised at 6 hpi, almost remained constant until 48hpi and then declined to its initial rate, while in susceptible cultivar (Super N3), elevation of CAT activity was continued to 24 hpi, remained constant until 72 hpi and then declined to its initial rate (Figure 3). According to the enzyme activity peak, elevation of CAT unlike to SOD was more than 1.5 fold (1.97 vs. 1.23 U) higher in the susceptible than in the resistant cultivar. Also, CAT activity was declined later (72h vs. 48h) in the susceptible cultivar which was much higher than in the resistant cultivar (Figure 3).

DISCUSSION

We recently conducted a comparative study on both cultivars “Green Magic” and “Super N3” along with 18 other greenhouse and outdoor cucumber cultivars in response to powdery mildew caused by *S. fuliginea* aiming to identify resistant and susceptible cultivars [21]. Wherein, the cultivars Green Magic and Super N3 were identified as extremely resistant and susceptible respectively. The cultivars were then chosen to investigate comparatively in terms of the factors studied here. Analysis of *PR-8* expression revealed that a higher level of *PR-8* transcript was expressed in the resistant

compared to the susceptible cultivar which is in accordance with the resistance of the cultivar against *S. fuliginea*. Chitin is a common material in the cell wall of fungal organisms including powdery mildew of cucurbits, thus chitinase hydrolyzing the pathogen cell wall is a quite relevant factor when studying resistance to the pathogen [7]. We clearly documented the upregulation of *PR-8* in the leaves of cucumber plants infected with powdery mildew caused by *S. fuliginea*. A strong relationship between resistance to the pathogen and upregulation of the *PR-8* gene can be deduced from the differences between the expression of this gene at 96 hpi in the resistance and the susceptible cultivars (Figure 1). These results agree with several other reports conducted on different plants infected with fungal pathogens e.g. barley infected with powdery mildew [8], cucumber infected with downy mildew [6] and muskmelon infected with *Fusarium oxysporum* [7]. In addition to fungal pathogens, induction of chitinase expression in relation with bacterial, viral and even abiotic stresses such as heat-shock have also been well documented [6,9]. Also, External applications of chitinases to control powdery mildew of strawberry by spraying on fruits and leaves [24] as well as to control the disease on barely by microinjection into coleoptile epidermal cells [25] have been reported.

Two antioxidant enzymes SOD and CAT showed contrary activities in the leaves of resistant and susceptible cultivars. As shown in Figures 2 and 3, SOD was more active and CAT was weaker in the resistant compared to the susceptible cultivar. Both CAT and peroxidase are antioxidant enzymes eliminating reactive oxygen species (ROSs). SOD catalyzes conversion of the superoxide free radicals (O^{2-}) to molecular oxygen and H_2O_2 and CAT catalyzes elimination of H_2O_2 during oxidative damages [10]. Thus, the patterns and levels of the activity of both enzymes in the resistant cultivar may be correlated with defense responses related to H_2O_2 accumulation. Different reasons for this argument are suggested including: i. accumulation of ROSs, e.g., H_2O_2 which is known as oxidative burst (OB) can be the earliest occurring events in plant host cells. The ROSs formed in this stage may act as antimicrobial agents, ii. in another different mode of action, H_2O_2 particularly takes part in cell wall fortification via cross-linking of the cell wall proteins, iii. H_2O_2 may serves as a substrate in cell wall apposition (papilla formation) [13], a strategy by which plants can restrict access of fungal pathogens to the plant protoplasts. Secondary metabolites with antimicrobial activity, accumulate in papillae structures. Also, rapid formation of such structures has been suggested to be correlated with enhanced resistance to pathogen penetration and vice versa [26]. Thus, successful cell wall-associated defenses such as papillae formation can resist the pathogen penetration at earlier stages and eliminate the need for costlier defense responses such as HR (hypersensitive response) cell death. Biotrophic parasites such as powdery mildew interacts with living plant cells by utilizing fine strategies for interacting with the host cell wall [27]. Thus, papillae formation can be an effective barrier against powdery mildew and other haustorium-forming fungi which have to penetrate cell wall to make feeding structures (haustorium) [28].

Additionally, H_2O_2 induces cell death and acts as a signal for induction of systemic defense responses [15]. Systemic acquired resistance (SAR) is another way by which plants respond to necrotizing pathogens leading to an enhanced resistance to the pathogen throughout the plant. It has been shown that salicylic acid acts by reducing CAT activity and this reduction acts as a second messenger in activating the expression of pathogenesis related genes [16]. Inhibition of spore germination of a number of fungal pathogens by H_2O_2 has also been reported [14]. In brief, considering the above mentioned H_2O_2 related defense responses on one hand and the activity of SOD and CAT enzymes in the leaves of the resistant cultivar (Green Magic) on the other hand, we may suggest plausible basic roles for H_2O_2 in the defense against the biotrophic powdery mildew causing agent *S. fuliginea*. Although, more biochemical investigations e.g. measuring H_2O_2 concentration changes during infection are required to prove this suggestion.

CONCLUSION

The results of the present study including analysis of PR-8 expression and determination of the activity of the two antioxidant enzymes i.e. SOD and CAT in the leaves of susceptible Super N3 and resistant Green magic cucumber cultivars clearly revealed different responses of the cultivars. The chitinase gene was highly expressed in both cultivars at 96 hpi, however the expression level was dramatically higher in the resistant compared to the susceptible cultivar implying a positive role of PR-8 in the resistance to *S. fuliginea*. In comparison, the resistant cultivar showed a higher level of SOD activity, while by contrast, a lower activity of H_2O_2 eliminating enzyme i.e.

CAT was observed which may suggest key roles for H_2O_2 in related resistance mechanisms.

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پاسخ خیار به *Sphaerotheca fuliginea*: تفاوت در فعالیت آنزیم‌های آنتی اکسیدانی و بیان ژن مرتبط با بیماریزایی در ژنوتیپ‌های حساس و مقاوم

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چکیده

سفیدک پودری یکی از زیانبارترین بیماری‌های خیار در سراسر جهان است. آگاهی دقیق از فرآیندهای زیستی که موجب مقاومت یا حساسیت گیاه به پاتوژن می شوند، می تواند مسیر را برای طراحی یک برنامه اصلاح مقاومت به پاتوژن هموار سازد. در مطالعه حاضر پاسخ به سفیدک پودری ناشی *Sphaerotheca fuliginea* به صورت مقایسه‌ای در دو واریته مقاوم و حساس خیار با استفاده از ارزیابی‌های مولکولی و بیوشیمیایی مورد بررسی قرار گرفته است. بیان ژن *PR-8* (کیتیناز کلاس۳) با استفاده از روش ریل تایم PCR و فعالیت دو آنزیم آنتی‌اکسیدانی سوپراکسیددیسموتاز و کاتالاز اندازه گیری و تجزیه وتحلیل شده است. نتایج نشان داد که بیان ژن *PR-8* در برگ‌های هردو رقم ۹۶ ساعت بعد از آلودگی افزایش یافت، اما این افزایش در رقم مقاوم ۶ برابر بیشتر از رقم حساس بود (۳۰۰ در مقابل ۵۰ برابر بیان نسبی نرمال شده). ممکن است این نتایج دلالت بر نقش کلیدی *PR-8* در مقاومت به این پاتوژن داشته باشد. آنزیم سوپراکسیددیسموتاز الگوی فعالیت یکسانی را در هر دو رقم نشان داد و در همان ساعت‌های اولیه آلودگی افزایش یافته و ۶ ساعت بعد از آلودگی اوج فعالیت خود را با فعالیت بیشتر در رقم مقاوم نشان داد. در مقابل، کاتالاز الگوی فعالیت متفاوتی را در دو رقم نشان داد و فعالیت بیشتری در رقم حساس داشت. نتایج پژوهش حاضر دید روشن‌تری نسبت به مکانیسم‌های احتمالی مقاومت به سفیدک پودری خیار ناشی از *S. fuliginea* ارائه می‌دهد.

کلمات کلیدی: خیار، کیتیناز کلاس۳، فعالیت آنتی اکسیدانی، ژن *PR-8*