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WOUND-INDUCED MOLECULAR ALTERATIONS AND ITS RELATIONSHIP TO PEROXIDASE ISOZYMES OF FUNGAL-INFECTED MELON GENERATIONS

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ABSTRACT

A transitory inhibition of either Prx_1 locus or its functional protein was observed in parental, F_1 and F_2 melon generations as a result to fungal infection with *Pseudoperonospora cubensis*. This locus was found to be activated again in leaf tissues of all generations either 48 hours after fungal elicitation or transcriptionally stimulated by unknown elicitors induced through 2 hours of wound treatment.

Rapid increase in peroxidase isozymes intensity after 24 hour of fungal inoculation was observed for resistant melon genotypes especially, P_1; PI 140471, F_1; P_1 x P_5 and P_2; PI 124111. Similar increments in isoperoxidase activity in resistant genotypes after 2 hours of wounding suggesting stimulation of the transcription level of all leaf isoperoxidases specially the four-banded Prx_2 locus. Meanwhile the susceptible genotypes; P_3; Honey dew and P_5; Ismaillawi did not show rapid changes in peroxidase isozymes.

Positive Spearman rank correlation coefficients were observed between downey mildew resistance and the highest isoperoxidase activity scores over all melon generations; 0.73 and 0.55, at 72 and 24 hours after fungal infection, respectively. The time course of wound induction of peroxidase activity suggests the inducibility of fungal resistance in leaf tissues by wound treatment. The results also suggest an indirect role for Prx_1 locus in inducing not only intermediate compounds, but also in stimulating transcription of genes involved in the plant defence system.

Keywords: *Cucumis melo*, *Pseudoperonospora cubensis,* Wound, Peroxidase, Isozymes, Germplasm.

INTRODUCTION

In a successful breeding program, germplasm characterized with resistance to any pathogen and or a high level of plant defence responses to environmental stress would be of great value in efficient programs for improving any plant species.

Of the many plant defence respon-ses to invasion by various pathogens, is the synthesis of a group of host-encoded proteins referred to as pathogenesis-related (PR) proteins. They were first discovered as polypeptides that accumulate in genotypes that respond hypersen-sitively to pathogen infection. Many PR proteins have antifungal activity (Bol *et al*., 1990). Recently, it has become known that the expression of some genes, that characterized with antifungal activity, are regulated by a large number of

hormonal and environmental signals which include wounding and fungal infection (Liu *et al*., 1994).

Major dominant gene resistance in plants is often expressed as a hypersensitive response. That is, in the vicinity of an infection, the cells of a resistant plant die, thereby, isolating the pathogen. Such cell necrosis is commonly associated with the accumulation of phenolic compounds and increased activity of oxidative enzymes. Although, both qualitative and quantitative differences have been found in a number of isozymes; phenoloxidase and peroxi-dase (Jennings *et al*., 1969; Retig, 1974) within the first twenty hours after inoculation of the pathogen, the activity of peroxidase enzymes consistently shows a rapid increase following fungal infection. Also, entirely new isozymes have occurred (Hislop and Stahmann, 1971). The positive associated between the rapidity of the response of peroxidase activity to fungal infection and disease resistance (Bi and Zhang, 1993) has led to the hypothesis that peroxidase and other oxidative enzymes may be an integral part of the host plants' defence mechanism.

However, plant elicitation with fungal infection causes a transitory inhibition of protein synthesis asso-ciated with early increase in ethylene concentration and then induction of a plant cell wall hydroxyl proline-rich glycoproteins (HRGP) begins (Roby *et al*., 1985). Either ethylene production or HGPR is also correlated with peroxidase isozymes and both are responsible of the mechanism of induced resistance to fungal infection (Stermer and Hammerschmidt, 1987).

Moreover, mechanical stress; wound, induces many molecular and biochemical changes in plant cells and tissues of which, it enhances cytokinin activity (Crane and Ross, 1986), induces both qualitative and quantitative changes in peroxidase isozymes, (Miller and Kelly 1989), increases peroxidases activity which some of them are involved in the oligogalcto-uronide-induced lignification (Svalheim and Robertsen, 1990). It also causes the induction of large amount of mRNA of 1-aminocyclopropane-1-carboxylic acid (ACC) synthase which is also auxin-induced, (Nakagawa *et al*., 1991). Also, wounding increases the produc-tion of ethylene which is different than endogenous ethylene production (Dunlap and Robacker, 1994).

In this investigation, the molecular polymorphism of peroxidase was used to detect the response of parental, F_1 and F_2 melon generations for two environmental elicitors which included fungal infection and wound treatment.

MATERIALS AND METHODS

Five parental genotypes of melon (*Cucmis melo* L.) represented by two plant introductions from the North Central Regional Plant Introduction Station, Ames, Iowa, USA; PI 140471 (Texas, USA) as P_1 and PI 124111 (India) as P_2 which is powdery mildew and the downy mildew resistant (Cohen *et al*., 1987), one foreign variety, Honey dew (USA) as P_3 and two local varieties obtained from the Horticultural Crops Research Institute, Cairo, Egypt; Ananas El-Dokki and Ismaillawi as P_4 and P_5, respectively, were used to achieve this study at El-Kassasein Horticultural Research

Station, Horticultural Crops Research Institute. These parents were sibbed for two successive seasons before its utilization for raising the crosses. Then, they were grown and crossed to obtain the three selective crosses P_1 x P_5, P_3 x P_2 and P_2 x P_4.

In January of 1997, F_1 seeds of all crosses were sown in plastic greenhouse and were grown and selfed to obtain the respective F_2 seeds. The sibbed seeds of the five parents, the F_1's as well as the F_2 (1 x 5), F_2 (3 x 2) and F_2 (2 x 4) were sown in January 1998 and were used to study the expression of peroxidase isozymes and their response to two environmental stress treatments. Both pathological and mechanical stress treatments were randomly distributed with three replications, in each set of parents, F_1 and F_2 plants of each cross. Other than stress treatments, normal agricultural recommended procedures were applied to melon plants in both greenhouse and field.

A. Pathological and wound treatments:

1- Fungal inoculation:

The inoculum was prepared from cucumber leaves, collected from different locations of Ismailliya governorate, which were already infected with *Pseudoperonospora cubensis*, the causal organisms of cucumber downy mildew disease. The detached leaves were then dipped in distilled water and gently shaked to wash off the exudates. The spore suspension was filtered through several layers of cheese cloth and its concentration was adjusted to 5 x 10^3 sporangia/ml using the haemocyto-meter slide. The inoculation proce-dures of melon plants were accomplished by spraying the plant according to

Thomas *et al*. (1987).

The first sample was taken immediately before inoculation, the other three samples were taken 24 hrs., 48 hrs and 72 hrs after inoculation time. Infection severity was estimated according to Horsfall and Barratt (1945).

2- Wounding treatment:

Plants were left to grow in the open field until the 2-3 true leaves stage, then an excision around the leaf edge in parental, F_1 and F_2 plants was done as wound treatment. Random samples were taken just before wounding of leaves. The other three samples were taken 2hrs, 6hrs and 24 hrs after wounding time, respectively.

B. Isozymes electrophoresis:

Three plants were randomly taken for each parent or F_1 population, while twelve randomly plants of each F_2 population were used as electrophoresis samples. The isozyme electrophoretic technique was applied on leaf tissue of each collected sample. Peroxidase isozyme system which was given the designation of EC 1.11.17 in the report of commission of enzymes (International Union of Biochemists, 1978), was screened in leaf tissues of both fungal-inoculated and wound-treated plants at the Biotech. lab., El-Kassasein Horticultural Research Station.

1- Enzyme extraction:

Equal weights of fresh samples were taken and crushed directly in an ice-cold (0-4°C) 1M tris buffer, pH 7.8. The enzyme extraction buffer and procedures were applied according to Tanksley and Orton (1983).

2- Gel preparation, sample loading and electrophoresis:

A stock of 30% acrylamide, N'N'-Bis methylene acrylamide stock (30% T and 2.67% C) was used for preparing a 15% discontinuous, non-dissociating polyacrylamide molds. TEMED and ammonium persulfate were added to initiate polymerization of acrylamide in a tris-borate buffer pH 8.6. The gel mixture was loaded in a 20 x 20 cm Bio-Rad PROTEAN II Vertical Slab Cell. A total of 40 μl (25 μl of sample in crushing buffer and 15 μl of 10% sucrose in 0.2% bromophenol blue solution) was loaded in each sample slot using a 0-200 μl changeable Eppendorf micropipette. Electrophoresis was continued until the bromophenol blue dye front has traveled to the end of the run. Six hours and half were needed for each run using constant voltage of 250 DC volts.

3- Staining and data collection:

For detection of isoperoxi-dases, gels were incubated for 30 minutes on 30°C in 0.01% benzidine hydrochloride in 1M sodium acetate solution of pH 4.7. Few drops of H_2O_2 were added immediately **(Guikema and Sherman, 1980)**.

The traveled distances by isozyme bands were recorded directly on graph paper as relative mobility values, Rm, then gels were photographed. The loci were repre-sented by a numerical subscript e.g. Prx_1, Prx_2, etc. in an ascending order from the fastest to the lowest moving anodal loci.

C. Statistical analysis:

Isozyme intensity scores of isoper-oxidase molecular forms were computed as 4.0 for the condensed band, 3.0 for the medium, 2.0 for the light, 1.0 for the faint one and a 0.0 estimate for the totally absent isoperoxidase band.

Spearman's rank correlation coefficients (Spearman, 1904) were computed to determine the associated relationships between low infection severity and changes in isozyme scores. Significance of rank correlation coefficients were tested according to Zar (1972).

RESULTS AND DISCUSSION

A. Isozyme polymorphism and fungal infection:

Peroxidase zymograms presented in Photographs (1,2 & 3) and figures (1, 2 & 3) showed that, in all crosses, the intensity of the four banded locus Prx_2 increased after fungal infection. This increments continued till 72 hours after infection especially in P_1 and F_2 generations of the cross P_1 x P_5 and in P_2, P_4 and F_1 of the cross P_2 x P_4. However, in addition to the stable increment of isozymes intensity, observed after infection in P_2 (PI 124111) of the cross P_2 x

P_4 , the Prx_1 locus expressed by the most anodal variant (3.4 cm), was found to be absent from all zymograms of P_1, P_2, F_1 and F_2 generations of the 24 hours samples. This locus was found to be activated again after 24 hours of fungal elicitation (Figs. 1, 2 and 3). This indicates that this locus might be suppressed shortly after fungal elicitation of leaf tissues especially in P_2, F_1 and F_2 of the cross P_4 x P_2 (Fig. 2), thereby, some fungal elicitors may be act as either protein transitory inhibitors or could be directly involved in turning of such locus. These results are in concordance with those obtained by Roby *et al*. (1985) who mentioned that the fungal elicitor causes an initial transitory inhibition of protein synthesis.

It is clear that activation of the Prx_1 locus, expressed with the isozyme 3.4 cm anodal to the origin, is demonstrated 48 hours after fungal inoculation regardless this activity will continue in generations of the cross P_4 x P_2 (Fig. 2) or will be suppressed shortly after that time as in crosses P_1 x P_5 and P_2 x P_4 (Figs. 1 & 3). New isozyms were also detected after fungal infection by Hislop and Stahmann (1971).

Data in Table (1) showed that P_1 (PI 140471), F_1 (P_1 x P_5) and P_2 (PI 124111), characterized with almost the lowest infection severity, had the highest positive changes in isoperoxi-dase scores at 24, 72 hours after inoculation with *P. cubensis* spores and according to the average of isoperoxidase score changes. This clearly indicates that both the Prx_1 and Prx_2 loci play and important role in the time course of infection.

Table 1: Isozyme activity score changes in different generations of three crosses among melon genotypes at 24, 48 and 72 hours after fungal infection and Spearman rank correlation coefficients (r_s) between low infection severity (IS) and changes in isozyme score.

Generation	I.S.	Isozyme score changes in isoperoxidases			
		24 hr	48 hr	72 hr	X
P_1 (PI 14047)	1.39	+18	2	+15	+10.3
P_5 (Ismaillawi)	9.62	0	-4	-2	-2
F_1 (P_1 x P_5)	1.93	+10	-4	+1	+2.3
F_2	1.40	+0.3	+1.8	+1.3	+1.1
P_3 Honey Dew	5.62	0	-8	-7	-5
P_2 (PI 124111)	2.79	+8	+8	+9	+8.3
F_1 (P_3 x P_2)	10.8	+3	+2	-2	+1
F_2	6.88	-4.3	-2.3	-1.3	-2.6
P_2 (PI 124111)	3.75	+4	+10	+2	+5.3
P_4 (Anannas El-Dokki)	5.16	+7	-1	+15	+7
F_1 (P_2 x P_4)	9.3	+2	+22	+1	+8.3
F_2	13.3	+1.5	-0.5	-3	-0.7
r_s		0.55 0.2<P<0.1	-0.01	0.73* 0.05<P<.02	0.53 .2<P<.10

*** Significant at 5% level.**

However, it is worthy to mention that the rapid increase in peroxidase isozymes activity after 24 hours of fungal infection, in resistant melon genotypes, was observed for P_1; PI 140471; F_1; P_1 x P_5 P_2; PI 124111, and the moderately resistant P_4; Anannas El-Dokki. Meanwhile, the susceptible genotypes P_4 and P_5 showed no rapid changes in the activity of the peroxidase isozymes. The consistent qualitative and quantitative changes in isoperoxi-dases, in P_1 and P_2, after 24, 48 and 72 hours of inoculation in this study were also observed within the first twenty hours after inoculation of the pathogen by Retig (1974). In resistant plants significant increase in soluble peroxidase levels have been detected by Nadolny and Sequeira (1980) within eight hours, by Jennings *et al*. (1969) and twenty four hours by Retig (1974). Similar rapid increases in peroxidase activity induced by fungal infection were also reported by Svalheim and Robertsen (1990). An increase of enzyme activity after fungal inocula-tion in cucumber was also observed by Zhang and Punja (1994).

A positive significant Spearman rank correlation coefficient (0.730) was observed between low infection severity and the highest changes in peroxidase isozyme activity scores at 72 hours after fungal inoculation. Moreover, a positive insignificant rank correlation coefficients, 0.55 and 0.53 were observed for both the activity scores 24 hours after inoculation and for the average activity scores over the three samples screened after *P. cubensis* inoculation. This indicates that pero-xidase activity is positively associated with downy mildew resistance and negatively correlated with infection severity (Table 1). Such PR proteins are also suggested to have antifungal activity (Bol *et al*., 1990). These results are correspondent with those obtained by Stermer and Hammerschmidt (1987) and Bi and Zhang (1993).

B. Isozyme polymorphism and wounding:

Peroxidase isozyme banding patterns for extracts of unwounded and 2, 6 and 24 hours after wounding, leaves of different genera-tions presented in Photographs 4, 5 & 6 and Figs. (4-6) clearly showed a rapid increase in bands intensity at 2, 6 and 24 hours after wounding especially in P_1, F_1 and F_2 in the cross P_1 x P_5 (Fig. 4) and in all generations of the crosses P_3 x P_2 and P_2 x P_4 as shown in Figs. (5 and 6), respectively. In addition, the appearance of the Prx_1 locus expressed with the molecular from of 3.4 cm anodal to the origin, in leaf extracts just 2 hours after wounding especially in P_1, F_1 and F_2 generations of the cross P_1 x P_5 and in P_2, F_1 and F_2 generations of the cross P_3 x P_2. Meanwhile, the slight expression, of this locus observed in all generations of the cross P_2 x p_4 (Fig. 6a) before wounding is found to be considerably increased after 2 hours of wounding treatments (Figs. 6 b,c and d). These results, also, suggest that wounding could be associated with an increase not only in the activity of the three or four molecular forms of the Prx_2 locus but also in the expression of the new peroxidase isozyme of Prx_1 at the 3.4 cm anodal to the origin which was absent before wounding treat-ment. This clearly indicated that wounding could produce different elicitors which, in turn, cause the transcription stimulation of Prx_1 and raising its level for Prx_2 locus in young leaf tissues.

Such qualitative and quantitative changes are, also, obtained by Miller and Kelly (1989). In this concern, Crane and Ross (1986) reported that wounding increases endogenous cytokinin, mRNA of ACC, (Nakagawa *et al*., 1991) and the induced ethylene (Dunlap and Robacker, 1994). Also, it is accompanied by several fold increases in the activity of pectin methylesterase and peroxidase induc-ing biochemical changes in major tissues. These results are in corres-pondence with those of Svalheim and Robertsen (1990). Thereby, this wound-induced peroxidase isoforms may be involved in induced-cell wall lignification and or in any subsequent induced fungal resistance. In this regard, Dean and Kuc (1987) concluded that systematically protec-ted leaves have a greater ability to lignify in response to wounding.

It is worthy to mention that similar trends were observed in inducing elicitors either 24 hours, after fungal inoculation in resistant germplasm or 2 hours after wounding treatment stimulating the single-banded Prx_1 or triggering the levels of the four-banded Prx_2 locus in leaf tissues. This clearly suggest that induced fungal resistance in leaf tissues could be obtained by wounding treatment. Similar, fungal and wounding resis-tance induced mechanisms are obtained by Liu *et al*. (1994). Moreover, it indicates that the molecular form of the Prx_1 locus has an important role in inducing not only intermediate compounds for lignin and or ethylene but also in stimulating transcription of genes involved in the plant defence system.

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التغيرات الجزيئية المستحدثة بالجرح وعلاقتها بأيسوزيمات البيروكسيديز فى أجيال الشمام المصابة بالفطر

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لوحظ حدوث تثبيط مؤقت للموقع الوراثى Prx_1 أو للبروتين الفعال الخاص به وذلك فى الآباء والجيلين الأول والثانى نتيجة للعدوى بفطر *Pseudoperonospora cubensis* ، وقد وجد أن هذا الموقع الوراثى يتم تنشيطه ثانية فى أنسجة الورقة فى كل من تلك الأجيال سواء بعد 48 ساعة من الإثارة بالفطر أو إنه قد يتم إثارته بمحثات غير معروفة تظهر خلال ساعتين من المعاملة بالجرح.

كما لوحظ حدوث زيادة سريعة فى ايسوزيمات البيروكسيديز بعد 24 ساعة من العدوى بالفطر للتراكيب الوراثية المقاومة من الشمام وخاصة (P_1 (PI 140471، F_1 (P_1 x P_5) ، PI) ، P_2(124111)، وقد لوحظت زيادات مماثلة فى نشاط أيسوزيمات البيروكسيديز فى التراكيب الوراثية المقاومة بعد ساعتين من المعاملة بالجرح مما يقترح حدوث إثارة لمستوى نسخ كل الإيسوزيمات فى الورقة وخاصة الموقع الوراثى Prx_2 ذو الحزم الأربع، بينما لم تظهر أية تغييرات سريعة فى ايسوزيمات البيروكسيديز فى التراكيب الوراثية الحساسة (P_3 (Honey dew, P_5 (Ismaillawi) .

وقد أوضحت الدراسة وجود معاملات ارتباط موجبة (معامل ارتباط سبيرمان للترتيب) بين المقاومة للبياض الزغبى وبين أعلى درجات لنشاط ايسوزيمات البيروكسيديز على مستوى كل أجيال الشمام إذ وجد أنها 73ر0، 55ر0 بعد 72 ، 24 ساعة من العدوى بالفطر على الترتيب، كما يقترح الجدول الزمنى لإثارة البيروكسيديز بالجرح أن المعاملة بالجرح يمكنها احداث إثارة لمقاومة الفطر فى أنسجة الورقة، كما تقترح النتائج وجود دور غير مباشر للموقع الوراثى Prx_1 فى إثارة ليس فقط مركبات وسطية ولكن ايضا فى تنشيط نسخ الجينات المشاركة فى نظام الدفاع النباتى.