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Proteins and resistance of wheat to stem rust: non-involvement of some serologically and electrophoretically determined proteins

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Leaf proteins of *Triticum dicoccum* cv. Khapli determined by the agar-gel-diffusion analysis and by electrophoresis were partly decomposed with simultaneous loss of resistance to *Puccinia graminis* f. sp. *tritici* race 11 in detached leaf cultures. However, abscisic acid treatment, which induced protein decomposition (senescence) of attached leaves similar to that induced by detachment, did not affect rust resistance. Furthermore, treatment with maleic hydrazide did not influence protein composition of primary leaves, but rust resistance was still lost in Khapli seedlings. It is concluded that, with the methods used in this study, protein changes in Khapli wheat leaves do not correlate with changes in resistance to stem rust.

INTRODUCTION

It is known that the detachment of Khapli wheat leaves induces susceptibility to stem rust in otherwise resistant tissues [*5*]. The wheat cv. Khapli, which is resistant to most of the races of stem rust (*Puccinia graminis* Pers. f. sp. *tritici* Erikss. et E. Henn.), regains its lost resistance or keeps the resistant character if the leaf cultures are held in a solution of kinetin or benzimidazole [*12*]. Leaf detachment is always correlated with senescence, but cytokinins, like kinetin or benzimidazole, inhibit the senescence effect in detached leaf cultures. Thus, there exists a correlation between senescence of Khapli wheat leaves and the lost rust resistance, on the one hand, and between juvenility of host tissues induced by cytokinins and resistance of detached leaves, on the other.

Applying serological methods, Lovrekovich & Király (cited by Goodman *et al.* [*6*]) have shown that at least two out of five antigens were lost in Khapli leaves 5 to 6 days after detachment, but no antigenic proteins were lost if the leaves were treated with cytokinins in the leaf cultures.

The object of the present investigation was to seek a correlation, if any, between the lost resistance and the lost antigenic proteins of Khapli wheat. The serological investigations mentioned above [*6*] were repeated. In addition we induced senescence in *attached* leaves by a senescence hormone, abscisic acid, to seek an eventual correlation between protein decomposition in senescent leaves and the shift of the resistant reaction to a compatible (susceptible) one. In further experiments we induced a compatible reaction in attached, resistant Khapli leaves by treating them with

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maleic hydrazide [*5*, *13*] and tried to find changes in leaf protein composition similar to that induced by detachment. Antigens determined by serological methods are most probably proteins. One can suppose that senescence induced by both detachment and abscisic acid as well as the treatment with maleic hydrazide may cause profound changes in leaf protein composition. Thus, after purification of leaf proteins, we investigated the protein composition of control and treated healthy Khapli leaves using the technique of polyacrylamide gel electrophoresis.

MATERIALS AND METHODS

Plant material

Seven-day-old seedlings of *Triticum dicoccum* Schubler cv. Khapli were treated with 50 mg/day maleic hydrazide supplied through the roots to plants or with 100 parts/10^6 water solution of abscisic acid by spraying the leaves three times daily for 8 days. Detached first leaves were taken from 11-day-old seedlings. The leaf surface was sterilized by a 0·5% solution of NaOCl for 2 min, then washed with sterile distilled water and floated in sterile Petri dishes on 20 ml sterile distilled water. The Petri dishes were held under ordinary greenhouse conditions in August and September.

Inoculations

In order to check the effect of different treatments on rust reaction, some of the plants were inoculated with uredospore suspension of *Puccinia graminis* Pers. f. sp. *tritici* Erikss. et E. Henn, race 11 and held in a moisture chamber for 24 h. The reaction types were determined 14 days after inoculation according to Stakman *et al.* [*16*]. Inoculations were made 6 days after beginning the treatment with maleic hydrazide, 1 day before or 2 and 4 days after the beginning of treatment with abscisic acid, and 4 days before detachment, respectively.

Preparation of antigens

Thirty g non-treated, healthy primary leaves were homogenized in 90 ml of 0·15 M-phosphate buffer (pH 7·0) with quartz sand in a cold room at 4 °C. The sap was expressed through four layers of cheesecloth and then centrifuged at 5500 *g* for 30 min in a Janetzki K 23 centrifuge at 0 °C. The supernatant was re-centrifuged in a preparative ultracentrifuge (MOM G 120) at 50 000 *g* for 1 h. The protein in the supernatant was precipitated by adding ammonium sulfate until 95% saturated, then centrifuged at 5500 *g* for 15 min at 0 °C. The pellet was dissolved in 0·1 M-phosphate buffer (pH 7·0) and dialyzed for 24 h against the same buffer at 4 °C. The protein content of the solution was determined by the method of Lowry *et al.* [*10*]. This protein antigen was injected into rabbits. In addition to protein antigen prepared in this way, leaf extracts purified by ultracentrifugation were also used as antigens in the agar-gel-diffusion test. Primary leaves, after different treatments, were homogenized and ultracentrifuged as mentioned above. In this case proteins were not precipitated; however, the supernatant of the ultracentrifuged leaf extract was used as antigen.

Immunization

Antisera were prepared in 3 to 4 kg rabbits by five intramuscular injections of the protein antigen at 2-week intervals. In fact, the rabbits were injected with 2·5-ml

portions of the mixture of 6·25 ml antigen (containing 1% protein), 12·5 ml of 0·9% NaCl, 5 ml $Al(OH)_3$ gel and 1·25 ml of 5% phenol into both hind legs. Ten days after the fifth injection the rabbits were bled through the carotid artery. The blood was collected through a sterile funnel in a sterile calibrated cylinder. The blood was allowed to cake overnight at 4 °C. Then the serum was drained off, and 0·1% merthiolate was added to avoid contamination.

Agar-gel-diffusion test

Fifteen g Bacto-agar (Difco) was melted in 1000 ml physiological saline solution, then 10 ml of 1% merthiolate and 10 ml of 0·1% trypanblue solutions were added. The pH was adjusted to 7·0. Fifteen ml agar was poured into each 10 cm diameter Petri dish. Wells 1 cm in diameter, spaced 1 cm between wells, were cut in the solidified agar. The 1 cm space between wells turned out to be the best in preliminary experiments for spur precipitin line test. The undiluted antiserum and different antigens were placed in the wells. The protein solution used for immunization or the ultracentrifuged leaf extracts were used as antigens. Petri dishes were incubated for 2 days at 37 °C or for 3 to 5 days at 22 °C and then the number and places of precipitation bands determined. Antisera titers were determined by the complement fixation reaction (cf. Gyarmati [7]).

Purification of proteins

Five-g samples of leaf tissue from the different treatments were homogenized with 15 ml 0·15 M-phosphate buffer (pH 7·0) and quartz sand in pre-cooled mortars at 4 °C. The sap was expressed through four layers of cheesecloth and then centrifuged at 5500 *g* for 30 min at 0 °C in a Janetzki K 23 centrifuge. The supernatant was then ultracentrifuged (50 000 *g* for 1 h) in a MOM G 120 analytical ultracentrifuge. The supernatant was passed through a Molselect G 25 column (26 × 1·8 cm) to remove the low molecular weight materials and eluted with 0·15 M-phosphate buffer (pH 7·0). Four-ml samples were passed through the column and 4-ml fractions were collected. The optical density of the different fractions was measured at 280 nm using a Unicam SP 800 spectrophotometer. The protein fractions were combined and concentrated by polyethyleneglycol (type 20 000).

Polyacrylamide gel electrophoresis

The method worked out by Davis [3] and Ornstein [11] was followed with minor modifications. Gels (7·5%) were prepared in 7·5 × 0·5 cm glass tubes. The electrophoresis was performed at pH 8·3 using a Shandon apparatus. From the concentrated protein samples 100, 200 and 400 μg proteins were applied and electrophoresed at 2 mA for 30 min, then at 4 mA/tube for 90 min. The gels were fixed with a mixture of ethanol : acetic acid (7%), 1 : 1 (v/v), for 2 h, and then stained with 1% amidoblack (Merck) dissolved in 2% acetic acid for 20 min and destained with 2% acetic acid for 4 days. Evaluation was made by scanning the gels in a Joyce Chromoscan densitometer.

RESULTS

The inoculation experiments supported the results known from the relevant literature [5]: detached Khapli wheat leaves floated on water or attached leaves sprayed with

maleic hydrazide changed their rust reaction from type 1 (resistant) to type 3 or 4 (susceptible). However, abscisic acid did not influence the resistant reaction (type 1) even after an 8-day treatment, in spite of the severe senescence effect (yellowing) which was experienced from the beginning of a 4-day treatment.

In the present study, just as in the former experiment of Lovrekovich & Király, quoted in reference [6], five protein antigens were determined by a serological analysis using the agar-gel-diffusion technique (Plate 1). The titer of the antiserum specific to attached, healthy leaves was 320, as determined by applying the complement-fixation reaction. As a result of detachment and treatment with abscisic acid, two precipitation bands (proteins) could not be detected in agar-gel-diffusion plates. In fact, the bands weakened and disappeared gradually according to the length of detachment or treatment with abscisic acid. This experiment has shown that, in spite of the similar senescence effects detected by the serological analysis mentioned above, leaf detachment and abscisic acid treatment influenced the rust reaction type of Khapli leaves differently. It would seem that the changes observed in protein antigens of the host do not parallel changes in host resistance to rust. This conclusion was supported by the experiments with leaves treated with maleic hydrazide (Plate 1).

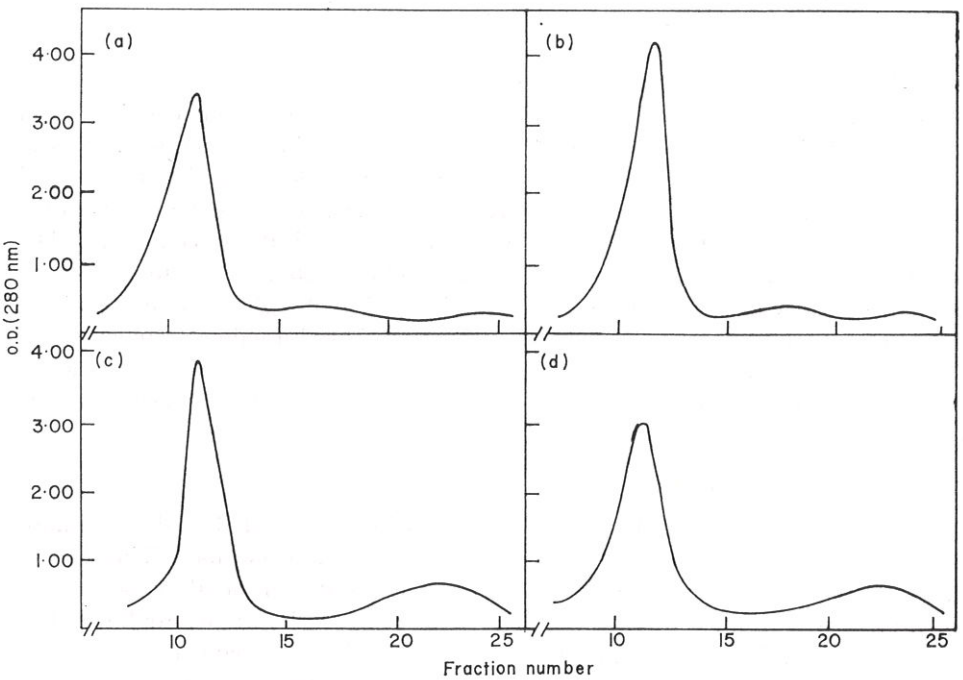

FIG. 1. Elution patterns of Molselect G 25 column (1·8 × 26 cm) gel filtration of extracts of Khapli wheat leaves. (a) Non-treated, attached leaf extract. (b) Extract from attached leaves treated with 50 mg maleic hydrazide/day for 8 days. (c) Extract from attached leaves treated with 100 parts/10^6 abscisic acid for 8 days. (d) Extract from detached leaves floated on water for 5 days. As regards leaf extraction and ultracentrifugation see PLATE 1. Four-ml samples were passed through the column and 4-ml fractions were collected after elution with 0·15 M-phosphate buffer (pH 7·0). The optical density of fractions was measured at 280 nm using a Unicam SP 800 spectrophotometer.

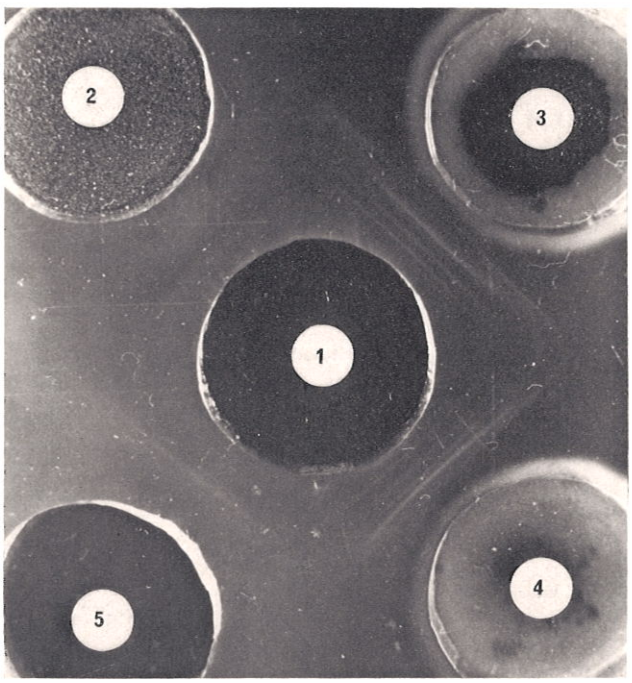

PLATE 1. Agar-gel-diffusion test for determining protein antigens in primary leaves of Khapli wheat seedlings. 1. Antiserum against attached leaves. 2. Non-treated attached leaf extract purified by ultracentrifugation (control). 3. Extract purified by ultracentrifugation from attached leaves treated with 50 mg maleic hydrazide/day for 8 days. 4. Extract purified by ultracentrifugation from detached leaves floated on water for 5 days. 5. Extract purified by ultracentrifugation from attached leaves treated with 100 parts/10^6 abscisic acid for 8 days. Thirty g leaf material was extracted with 90 ml of 0·15 M-phosphate buffer (pH 7·0), then centrifuged at 5500 *g* for 30 min. The supernatant was ultracentrifuged at 50 000 *g* for 1 h. The antiserum was prepared against the non-treated primary leaf proteins extracted and ultracentrifuged as mentioned above and then precipitated by ammonium sulfate from the supernatant.

[*facingpage 140*]

The latter treatment is able to change the resistance reaction of attached Khapli leaves to susceptibility, and still there is no loss in leaf proteins characterized by precipitation bands in agar-gel-diffusion plates.

The elution patterns of gel filtration of different leaf extracts (optical density at 280 nm) are seen in Fig. 1. It is obvious that as a result of leaf detachment or treatment with abscisic acid the optical density of fractions 19 to 25 is increased at 280 nm. This might indicate the presence of peptides with a low number of amino acid residues and also suggests that there is extensive protein decomposition. Fractions 7 to 15 were combined in each case and concentrated by polyethyleneglycol to a volume of 3 ml. The electrophoretic gel patterns of leaf proteins of different samples are seen in Fig. 2. Results with acrylamide gel electrophoresis are similar to those obtained with the agar-gel diffusion serological test. Treatment with maleic hydrazide, which caused a breakdown of resistance in attached leaves, did not change protein composition as compared to the control (Fig. 2). The action on protein decomposition of leaf detachment and abscisic acid treatment is almost identical (Fig. 2). There was not a single peak which decreased as a result of leaf detachment and, at the same time, was unchanged upon abscisic acid treatment. Thus, changes in leaf proteins

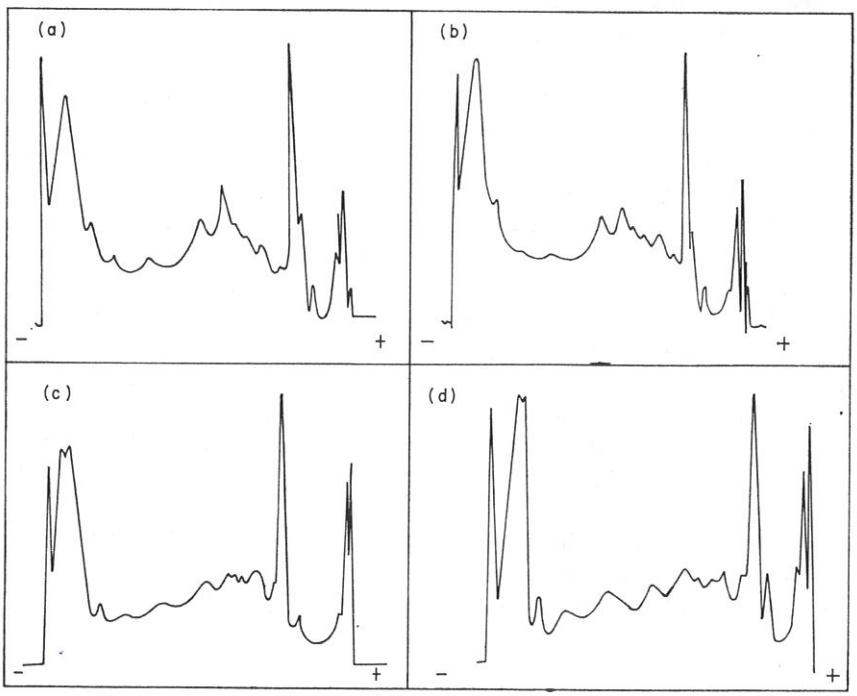

FIG. 2. Joyce Chromoscan densitometer scans of polyacrylamide gels after electrophoresis of proteins (200 μg) of Khapli wheat leaf samples. (a) Non-treated leaf proteins (control). (b) Proteins from attached leaves treated with 50 mg maleic hydrazide/day for 8 days. (c) Proteins from attached leaves treated with 100 parts/10^6 abscisic acid for 8 days. (d) Proteins from detached leaves floated on water for 5 days. The electrophoresis was performed at pH 8·3 using a Shandon apparatus, and stained with amidoblack.

determined by polyacrylamide gel electrophoresis were not in correlation with changes in rust resistance. The electrophoretic investigation was conducted also with crude leaf extracts to rule out the possibility of losing important protein components during procedures of purification. However, the electrophoretic gel patterns of fresh leaf extracts were identical with those obtained with purified leaf proteins.

DISCUSSION

Regarding the mechanism of plant disease resistance to fungal infections, it was shown during the past decade that a series of phenomena thought to be in a cause and effect relationship with resistance are only correlative in nature. Even the rôle of the hypersensitive reaction (tissue necrosis) was questioned [*1*, *8*]. Littlefield [*9*] called attention to the importance of rust resistance phenomena other than the hypersensitive reaction. It was also shown by careful investigations [*2*, *14*] that phenolics do not have a rôle in wheat rust resistance either as preformed resistance factors or as post-infectionally induced agents. The rôle of peroxidase in rust resistance was also re-investigated by Seevers *et al.* [*15*]. They concluded that the high peroxidase activity in the incompatible (resistant) host is a consequence of other unknown biological events and, indeed, is a secondary effect, not a primary determinant, in resistance. Activated peroxidase, polyphenoloxidase and accumulation of the phytoalexin rishitin proved to be consequences, not causes, of resistance of potato to *Phytophthora infestans* [*4*].

The present investigations demonstrated that some of the leaf proteins, identified either by serological or by electrophoretic methods, cannot be related to stem rust resistance. It is true that Khapli wheat proteins, which are lost together with resistance to rust as a consequence of leaf detachment, are not decomposed in the presence of cytokinins, and that these keep detached leaves resistant in culture [*6*]. However, abscisic acid which induced senescence (protein decomposition) similar to that induced by leaf detachment did not affect rust resistance. On the other hand, disease resistance was lost in leaves treated with maleic hydrazide but leaf protein composition was not affected as determined either by the agar-gel-diffusion analysis or by electrophoresis. In conclusion, with the methods outlined in this investigation, it was not possible to relate protein changes of Khapli wheat leaves to changes in resistance to stem rust. It would seem that the primary determinant(s) of wheat resistance to stem rust still remains unknown.

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