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A Putative Viral Resistance-Connected Protein Isolated from Potato Cultivar Sante Resistant to PVY^{NTN} Infection

By

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With 3 Figures

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Summary

GRUDEN K., ŠTRUKELJ B., RAVNIKAR M. & HERZOG-VELIKONJA B. 2000. A putative viral resistance-connected protein isolated from potato cultivar sante resistant to PVY^{NTN} infection. – *Phyton* (Horn, Austria) 40 (1): 191–200, 3 figures. – English with German summary.

The causal agent of potato tuber ring necrotic disease of potato virus Y^{NTN} (PVY^{NTN}) is a recently recognized and highly aggressive isolate of the PVY^N strains. The protein composition of infected and control plants of some potato (*Solanum tuberosum* L.) cultivars that differ in their susceptibility was compared. The differences in protein patterns become visible four days after infection and pronounced two weeks after infection. Most changes in protein composition occurred in the very sensitive cv. Igor as shown both by SDS polyacrylamide gel electrophoresis as well as by isoelectric focusing. Nine induced proteins were clearly

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recognised and decrease in concentration of some proteins was also observed. Proteins with increased expression were also detected in infected tissues of virus tolerant cvs. Desirée and Pentland squire. Interestingly, no changes in protein expression was observed in a sample of infected potato cv. Sante which is highly resistant to the virus. However, a constitutive protein of M_{app} 43000 was shown to be present in significantly higher concentrations compared to other cultivars. The putative resistance-connected function of this particular protein was confirmed in another resistant cultivar – cv. Carlingford in which the amounts of the 43000 protein are comparable to those in cv. Sante. Preparative SDS polyacrylamide gel electrophoresis was used to isolate the protein and its N-terminal amino acid sequence was determined.

Zusammenfassung

GRUDEN K., ŠTRUKELJ B., RAVNIKAR M. & HERZOG-VELIKONJA B. 2000. Ein vermutlich mit der Virusresistenz in Zusammenhang stehendes Protein wurde von der gegen PVY^{NTN} Infektion resistenten Kartoffelsorte Sante isoliert. – Phytion (Horn, Austria) 40 (1): 191–200, 3 Abbildungen. – Englisch mit deutscher Zusammenfassung.

Die verursachende Substanz der Kartoffelknollenringnegrosenkrankheit, welche durch das Kartoffelvirus Y^{NTN} (PVY^{NTN}) verursacht wird, wurde kürzlich entdeckt und ist ein höchst aggressives Produkt der PVY^N Linien. Bei einigen Kartoffelsorten (*Solanum tuberosum* L.), die sich in ihrer Empfindlichkeit unterscheiden, wurden die Proteinmuster infizierter und Kontrollpflanzen verglichen. Unterschiede im Proteinmuster konnten 4 Tage nach der Infektion beobachtet werden, deutlich jedoch waren sie 2 Wochen nach der Infektion. Die meisten Veränderungen in der Proteinzusammensetzung zeigten sich in der sehr empfindlichen Varietät Igor, was sowohl mit SDS Polyacrylamidegelelektrophorese nachgewiesen wurde, als auch mittels isoelektrischer Focussierung. Neun induzierte Proteine waren deutlich zu sehen, aber auch eine Konzentrationsabnahme einiger Proteine konnte beobachtet werden. Auch in Geweben der virustoleranten Varietäten Desirée und Pentland squire konnten Proteine mit stärkerer Ausbildung beobachtet werden. Interessanterweise konnten keine Veränderungen in der Proteinexpression bei Proben der infizierten, aber stark virusresistenten Sorte Sante festgestellt werden. Jedoch war ein normalerweise vorhandenes Protein von M_{app} 43000 in signifikant höheren Konzentrationen gegenüber den anderen Sorten zu beobachten. Die vermutlich mit der Resistenz verbundene Funktion dieses speziellen Proteins wurde auch mit einer anderen resistenten Sorte Carlingford bestätigt, in welcher die Menge des 43000 Proteins vergleichbar mit jener in der Varietät Sante war. Mittels präparativer SDS Polyacrylamidegelelektrophorese wurde das Protein isoliert und seine N-terminalen Aminosäuresequenzen wurden bestimmt.

Introduction

Potato virus Y^{NTN} (PVY^{NTN}) is a new strain in the family of Potyviridae first found in 1984 in Hungary. The main characteristic of virus PVY^{NTN} infection is the appearance of ring shaped necrosis on the

tubers (potato tuber ring necrotic disease) in addition to the symptoms of strain PVY^N infection (LE ROMANCER & al. 1994). It is spreading very rapidly, causing a severe crop yield reduction in cultivation of tomato, tobacco and pepper in addition to potato (KUS 1995). Potato cultivars differ in their susceptibility to the virus PVY^{NTN}. Cv. Igor, for example, is highly sensitive, developing strong symptoms of the disease that make the potatoes unmarketable. Producers have therefore completely abandoned the cultivation of this otherwise very popular cultivar in Slovenia. Some cultivars like cv. Desirée and cv. Pentland squire are more tolerant. The plants can be infected but do not develop severe symptoms. Finally, a few cultivars like cv. Sante and cv. Carlingford are resistant to the infection. Natural resistance of plants to particular pathogens is conferred by resistance genes (HAMMOND-KOSACK & JONES 1996, ROSSI & al. 1998). At least 12 resistance genes were isolated from different *Solanaceae* wild species for resistance to PVY (COCKERHAM 1990). The N resistance genes control necrotic reaction leading to hypersensitive response and the R genes control extreme resistance (VALLEJO & al. 1995). Ry(sto) gene was isolated from *Solanum stoloniferum* in connection with the resistance reaction of this species to PVY and tobacco etch virus (HINRICHS & al. 1998), and was incorporated into some cultivars like cv. Sante by classical breeding. The resistance of cv. Carlingford is different since it is polygenic in nature. The resistance genes involved in ligand-receptor mechanisms for pathogen recognition and the induction of signal transduction pathways code for proteins showing some common structural features like leucine rich repeat motif and nucleotide binding site (BAKER & al. 1997). However, the exact molecular and biochemical processes under the control of resistance genes are largely unknown. In this study the total soluble protein composition in leaves of different potato cultivars was analysed in control and PVY^{NTN} infected plants. Five cultivars that respond differently on interaction with the virus were chosen for the experiment, in an attempt to find protein(s) involved in resistance mechanism of potato against the virus.

Materials and Methods

Plant Material

Potato (*Solanum tuberosum* L.) cultivars Igor, Desirée, Pentland squire, Sante and Carlingford were grown in pots in growth chambers (Kötterman KG 2730) at constant temperature 20 ± 1 °C, humidity 80 % and illumination $5.5\text{--}10.1$ W/m² 12h per day. Plants were obtained by micropropagation using stem node culture. Four weeks after transplantation to soil one third of plants were leaf inoculated with sap from PVY^{NTN} infected plants, one third with sap from healthy plants and the last third of plants was left untreated. The plants were harvested 4 and 14 days after infection, immediately frozen in liquid nitrogen and stored at -70 °C.

SDS polyacrylamide gel electrophoresis and isoelectric focusing analysis

Plant leaves were homogenised first by mortar and pestle and then using a Potter homogeniser. The plant juice was squeezed out and centrifuged (12000 rpm, 10 min, 4 °C). Protein concentration in extracts was determined as described by BRADFORD 1976. SDS polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions was prepared as described by AUSUBEL & al. 1998. 30 µl of plant extract was loaded on 12 % polyacrylamide gels, 0.75 mm thick and 40 cm long. After separation, proteins were detected by silver staining (OAKLEY & al. 1989). The resulting gels were scanned and analysed by Molecular Analysts/PC software (BioRad, USA). Isoelectric focusing gels were prepared as described by AUSUBEL & al. 1998. A pH gradient from 3–10 was formed using carrier ampholytes (Pharmacia LKB, Sweden). 100 µl of plant extract was loaded on the gel and proteins were separated in 3000 volthours at 15 W. After electrophoresis the gel was stained with Coomassie blue.

Protein isolation and microsequencing

The first step in separating proteins from the leaf extract of cv. Sante infected by PVY^{NTN} was preparative SDS-PAGE using the same gel length as described above. The position of proteins was determined by silver staining part of the gel. The gel band corresponding to 43000 protein in the stained gel was cut out from the unstained part of the preparative gel. Protein(s) were eluted from the gel in distilled water and concentrated by precipitation with acetone. The samples obtained from three preparative electrophoreses were combined and reloaded on SDS-PAGE. This gel was left to run twice as long as normal to get maximal separation of the 43000 protein and possible contaminating proteins. Protein(s) in the gel were blotted to PVDF membrane (Immobilone-P, Milipore, USA) using Tris/Glycine/Methanol buffer (pH 9.2) at 1.2 mA/cm² for 1.5 h (AUSUBEL & al. 1998). The membrane was first extensively washed with distilled water and then stained using Coomassie blue to determine the position of the desired protein band and to determine the approximate amount of the protein (LEGENDRE & MATSUDAIRA 1989). The band was cut out and the protein submitted to Edman degradation. The N-terminal amino acid sequence was compared to the sequences deposited in protein and nucleic acid databases using the internet version of FASTA and BLAST software (ALTSCHUL & al. 1990).

Results

Protein composition in leaves of different potato cultivars after infection with PVY^{NTN}

Protein composition of extracts prepared from potato cvs. Igor, Pentland squire, Desirée and Sante leaves inoculated with sap from PVY^{NTN} infected plants and from healthy plants was compared. Protein concentration in leaf extracts of healthy plants was approximately 60 µg/ml in all analysed cultivars. Interestingly, total protein concentration was almost three-times higher in PVY^{NTN} infected leaf extract of cv. Igor (170 µg/ml), while in the extracts of infected cvs. Pentland squire,

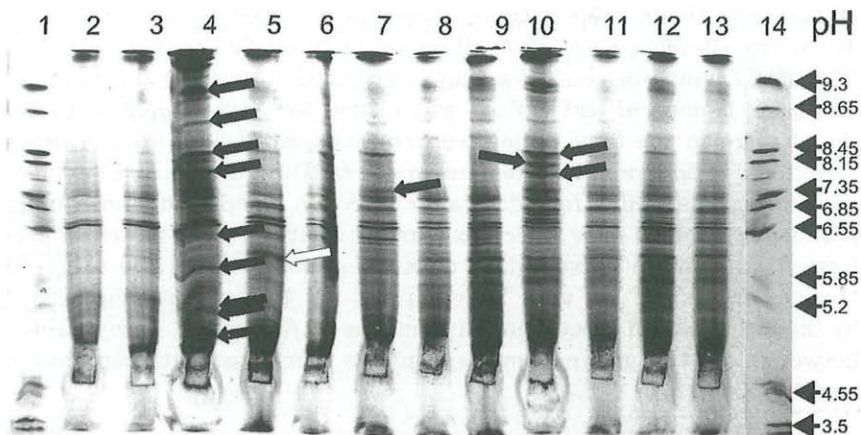


Fig. 1. Isoelectric focusing of different potato protein extracts. 1) and 14) standards, 2) leaves of normal, 3) control and 4) infected plants of cv. Igor, 5) leaves of normal, 6) control and 7) infected plants of cv. Pentland squire, 8) leaves of normal, 9) control and 10) infected plants of cv. Desirée, and 11) leaves of normal, 12) control and 13) infected plants of cv. Sante. Black arrows indicate the induced proteins, white arrows indicate protein breakdown.

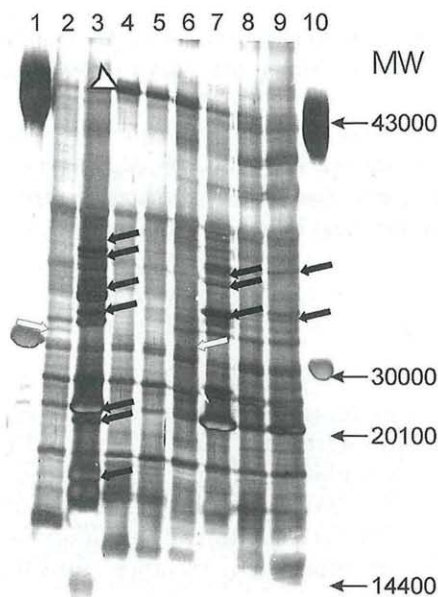


Fig. 2. Analytical SDS-PAGE of leaf protein extracts from different potato cultivars. 1) and 10) MW standard, 2) control and 3) PVY^{NTN} infected cv. Igor, 4) control and 5) PVY^{NTN} infected cv. Sante, 6) control and 7) PVY^{NTN} infected cv. Desirée, 8) control and 9) PVY^{NTN} infected cv. Pentland squire. Black arrows indicate the induced proteins, white arrows indicate protein breakdown. → indicates the position of 43000 protein.

Desirée and Sante protein concentration was raised for approximately 30 % (to 90–100 µg/ml). Two different methods for protein analysis, SDS-PAGE and isoelectric focusing, were used to analyse the samples. The same volume of leaf extract was loaded for each sample to follow the changes in the total protein concentration as well as up and down regulation of individual proteins expression. The differences in protein pattern become visible four days after infection (results not shown) and pronounce 14 days after infection. Further analyses were therefore performed on samples harvested 14 days after infection. All the observed changes are due to the viral infection and not to the wounding caused by the mechanics of inoculation of plants as no differences were detected between normally grown plants and plants inoculated with sap from a healthy plant (Fig. 1).

Figure 1

The reaction to infection was the most intense in cv. Igor as was indicated also by increase in total protein concentration. The expression of at least nine different proteins was induced or enhanced after infection as shown by isoelectric focusing (Fig. 1) as well as by SDS-PAGE (Fig. 2). Higher concentration of these proteins is not just the consequence of general raise in protein concentration in infected potato cv. Igor as their ratio towards other proteins in the sample is also raised. The approximate molecular weight of induced proteins was calculated and compared to molecular weight of viral proteins (Table 1). Viral proteins with molecular weight in the analysed range are coat protein and P1 protein, both with approximate molecular weight (MW_{app}) 30000. The induced proteins of 31000 and 31600 could represent viral proteins. All other induced proteins, consequently, are the products of potato genes. Some proteins are present in lower concentrations in infected sample, indicating protein breakdown or down regulation on the gene level (Fig. 2).

Figure 2

New or enhanced protein bands also appeared in the samples of infected tissue of cvs. Desirée and Pentland squire (Fig. 1 and 2, Table 1). No differences were observed in protein samples from infected and non-infected potato cv. Sante using either SDS-PAGE or isoelectric focusing. On the other hand, a protein with M_{app} 43000 is constitutively expressed in cv. Sante in significantly higher concentrations than in other cultivars (Fig. 3).

Table 1. Comparison of approximate molecular weight of induced proteins in different potato cultivars and viral proteins with MW in analysed range.

induced proteins (MW _{app})		viral proteins (MW _{app})	
Igor	Pentland squire	Desirée	
17800			
19000			
19500			
31000			30000
31600			(P1, CP)
	31800	31800	
	34700		
	35000		
35500			
		35700	
36300			

Computer analysis of the protein band patterns from SDS-PAGE

The intensity of the 43000 protein band in different samples of cvs. Sante, Igor, Pentland squire, Desirée was computer analysed. In addition another resistant cultivar, cv. Carlingford, was chosen for the analyses to check the relevance of 43000 protein for potato resistance against PVY^{NTN}. Part of the gel with proteins of M_{app} from 40000 to 45000 was scanned and intensity of the bands was determined using 1-D profile analysis. Results presented in Fig. 3 represent the mean values obtained in three separate experiments.

Figure 3

The amount of 43000 protein did not differ significantly when comparing infected and noninfected samples of all five cultivars. The concentration of protein in the control and infected leaves of cv. Sante however was three times higher than in cv. Desirée and more than ten times higher than in cv. Igor and cv. Pentland squire. The amount of the 43000 protein in cv. Carlingford was comparable to the amounts in cv. Sante.

N-terminal amino acid sequence of 43000 protein

The constitutively expressed 43000 protein in cv. Sante was isolated by two consecutive separations on SDS-PAGE, electroblotted to PVDF membrane and subjected to Edman degradation. The N-terminal sequence was determined to be S-E-K-K-T-L-N showing that the sample consists of only one protein.

The only protein with significant similarity to the obtained sequence found through database search was β_3 chain of Na⁺/K⁺ transporting ATP-

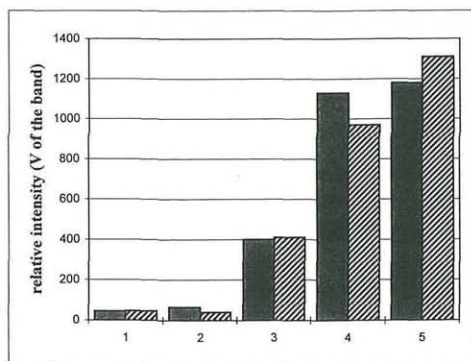


Fig. 3. 1-D profile analysis of 43000 protein band intensity in different leaf extracts. 1) cv. Igor, 2) cv. Pentland squire, 3) cv. Desirée, 4) cv. Sante, 5) cv. Carlingford. Grey boxes represent samples from control plants, hatched boxes samples from PVY^{NTN} infected plants.

ase from guinea pig. The 85 % identical sequence in ATP-ase starts four amino acids after the translation initiation codon.

Discussion

Differences in the level of resistance to infection with PVY^{NTN} between different potato cultivars have been described (KUS 1995). The protein composition in leaves of control and infected plants was followed to get an insight into the plant-pathogen interaction and the defence mechanism of resistant cultivars Sante and Carlingford. The changes in protein expression and protein degradation in cvs. Igor, Desirée and Pentland squire possibly indicated pathological processes and/or defence reaction as a consequence of infection. Interestingly, all proteins that are induced are not the same if comparing Desirée, Pentland squire or Igor. The only exception is the 31800 protein that is induced in cvs. Desirée and Pentland squire. In resistant cultivars Sante and Carlingford no reaction to viral infection was detected. It was similarly shown previously that after PVY^{NTN} infection the metabolism of cytokinins switches from synthesis of active forms to synthesis of inactive forms in susceptible cv. Igor while in cv. Sante no changes were observed (DERMASTIA & al. 1995). The exact mechanism of extreme resistance is not established. It could be possible that the constitutively expressed 43000 protein is a part of resistance mechanism preventing the viral infection and/or viral spread as it is expressed in significantly higher concentration in both cv. Sante and cv. Carlingford than in susceptible cultivars. The stretch of seven amino acid sequence determined is very short. The alignments of short protein N-terminus to sequences in the databanks are often not very relevant as the

conserved regions are mostly not located in the N-terminus of the protein. A complete primary structure will be needed for a relevant database search. The exact function of the 43000 protein in defence against PVY^{NTN} as well as some other pathogens is yet to be determined in bioassays with transgenic plants.

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Recensio

LAUNERT Edmund 1998. Biologisches Wörterbuch. Deutsch–Englisch, Englisch–Deutsch. – Gr. 8°, 739 Seiten, geb. – UTB für Wissenschaft: Große Reihe [letzteres steht weder auf Umschlag noch Titelblatt]. – Verlag Eugen Ulmer Stuttgart. – DM 78,-. – ISBN 3-8001-2577-3.

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Für den Rezensenten hat sich das vorliegende Wörterbuch schon bewährt, denn er fand darin bei der Arbeit in den letzten Monaten mehrfach englische Äquivalente, die in den Standard-Wörterbüchern, die man als Systematiker auf dem Schreibtisch hat, nicht enthalten waren. Außer für Fachwissenschaftler müßte dieses umfangreiche Wörterbuch aber auch für Übersetzer sehr nützlich sein; bei völlig fehlender biologischer Fachkompetenz werden letztere allerdings dennoch nicht ohne Wörterbücher bzw. Lexika mit Erläuterungen auskommen.

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