Chitinases and Endoglucanases Synthesized in the Infected Barley Leaves in the Powdery Mildew Period Sporulation

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Abstract

In our work, we represent genetic, molecular and biochemical changes induced in the infected barley leaves induced in the period pathogen sporulation. We studied a presence of acidic β-1,3-endoglucanases and chitinases in an intracellular fluid isolated from primary barley leaves infected with two powdery mildew pathotypes carrying various virulence genes. In the period of pathogen sporulation acidic β-1,3-endoglucanases (Glu) and chitinases (Chi) isozymes were synthesized in two main groups. Non-specific, high molecular group of these enzymes with Mr > 200 kDa did not differ genetic differences between host and pathogen. Specific, low molecular forms reflected both genetic background of host and pathogen as well as biochemical status of the disease. Low molecular acidic Glu had Mr from interval 9–12 kDa. Analogical situation was observed for low molecular chitinases. Their molecular masses were from interval 14.4–15.5 kDa. Intracellular fluid from the primary leaves of the healthy plants did not contained detectable amount of these enzymes. Appearance of low molecular forms of Glu and Chi is discussed.

Keywords: barley; acid proteins; chitinases; acid endoglucanases; powdery mildew

INTRODUCTION

Hydrolytic enzymes play very important task by host-pathogen interactions. They are synthesized very quickly after infection both host and pathogen but mode of their action is opposite. Many from them are classified between PR- and DR-proteins (pathogen-related and defense-related proteins, VAN LOON 1985). PR- and DR-proteins affect ion fluxes on the membrane system, oxidative processes leading to the oxidative burst, phosphorylation state of regulatory proteins, induce deposition of callose, lignin and suberin at a site of infection (HARTLEB *et al*. 1997). Their spectrum strongly depends on both resistance genes of the host genome and pathogen virulence/avirulence genes as well as on the onthogenetic phase of pathogen development (HLINKOVÁ *et al*. 1995). A large group of these hydrolytic enzymes create chitinases (EC 3.2.1.14) and glucanases (EC 3.2.1. 39). They are divided into 5 families regulated with *ypr* 2, 3, 4, 8 and 11 genes (VAN LOON & VAN STRIEN 1999). β-1,3-glucanases and chitinases were identified for both acid and basic region of protein spectra. Their patterns differ for extra and intracellular extracts (REPKA *et al*. 1997; MEMELINK *et al*. 1990). Chi and Glu represent structurally diverse group of enzymes, which differ in their physical properties, activities and location in plant compartment (SHINSHI *et al*. 1990). Besides defense response in infected plants they are also exhibit during early phase of plant development (WU *et al*. 2001; REZZONICO *et al*. 1998; VÖGELI-LANGE *et al*. 1994). Gene expression for Glu and Chi can be inhibited gibberellin and abscisic acid (WU *et al*. 2001; LEUBNER-METZGER *et al*. 1995).

β-1,3-glucanases (*ypr2*) hydrolyse 1,3-D-glucosidic bound in β-1,3-glucan. On the base of the amino-acid sequence are classified into V classes. Glu I are nonspecific and they are localized in plant vacuoles. Glu II and III are transported into extracellular space. About Glu IV and V knows less. Their anti-fungal effects are different (CORNELISSEN & MELCHERS 1993; PAYNE & RYALS 1990).

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Chitinases [1,4-(N-acetyl-β-D-glucosamide)-glycanohydrolase] hydrolyse chitin, a linear polymer of β(1→ 4)-N-acetylglucosamine (GlcNAc) (TRUDEL & ASSELIN 1989; LEUBNER-METZGER & MEINS 2000). Chi-s degrade double bounds between C = C of two following N-acetylglucosoamine in chitin on N, N`-diacetylchitobiose and higher oligomers. Hydrolysis can be carried out endo- or exohydrolytic pathway (SHINSHI *et al*. 1990). Plant Chi are divided into VII classes, which are coded genes *ypr3(Chi a), ypr4(Chi d), ypr8(Chi b*) and *ypr11(Chi a)* (VAN LOON & VAN STRIEN 1999). Extracellular Chi-s have antifungal effect (SELA-BUURLAGE *et al*. 1993). Glu and Chi have a synergic effect and their overproduction in the transgenic plants have caused higher level of resistance against to pathogens (TRUDEL & ASSELIN 1989; MELCHERS & STUIVER 2000; TAIRA *et al*. 2002). Chi-s and Glu-s of dicotyledonous plants infected with pathogens are better studied as monocot.

The aim of our work was to find differences between acidic intracellular β-1,3-glucanases and chitinases synthesized in the primary barley leaves of various genotypes infected by two strains of powdery mildew carrying different avirulence/virulence genes in the period of pathogen sporulation.

MATERIAL AND METHODS

Eight days aseptically cultivated (T = 20°C, irradiance 800 $\mu M.m^2/s$, daylight regime 16/8 h) young plants of three isogenic lines of barley (*Hordeum vulgare* L.*)* cv. Pallas [P02(*Ml-a3);* P04B(*Ml-a7);* P10(*Ml-a12*)] were used to the study of their sensitivity and molecular – genetic characteristics after infection with two different pathotypes of powdery mildew [*Blumeria graminis (*DC*)* Speer f.sp.*hordei*] in the period pathogen sporulation. As a susceptible control cv. Dvoran, without resistance genes from *Ml-a* locus was used (Table 1).

Plants were inoculated with pathogen RU-3 (powdery mildew strain of TU München-Weihenstephen isolated Drs. FISCHBECK and WOLFE) and Sk-5/11 (powdery mildew strain of Department of Genetics CU University – Dr. PLESNÍK). Basic genetic characteristics of pathogens as well as their virulence ability were determined according to SCHWARZBACH and FISCHBECK (1981) represented Table 2.

Eight days after inoculation, primary leaves were detached from the inoculated and control plants. Leaves were cleared from pathogen mycelia and cut on the non-infected (NIP) and infected parts (IP). Leaves from healthy, non-inoculated plants as a control were used. The extracellular fluid was extracted from leaves segments according to REPKA *et al*. (1993). After centrifugation extracellular fluid was transferred in Eppendorf tube and frozen by –20°C. Leaf material was then homogenized in liquid nitrogen and extracted 24 h by 4°C in 0.1M Na-phosphate buffer pH7, containing 6μM NaCl, 0.5 mM EDTA, 1μM $MgSO_4$ and 0.25% glycerol (HLINKOVÁ & ONDŘEJ 1994). Proteins were quantified with bovine serum albumin as a standard by the method of BRADFORD (1976). Separation of proteins was done by non-denaturating (A-PAGE) and denaturating (SDS-PAGE) conditions on 12.5% discontinuous polyacrylamide slab gels using Laemmli's buffer system (LAEMMLI 1970). Proteins were visualized with silver nitrate staining our modification of NESTERENKO *et al*. (1994) method. Molecular mass of proteins for SDS-PAGE was determined from calibration curve for 10 kDa protein leader kit of Gibco BRL (Life Tech., USA). Molecular mass of acid nondenaturated proteins (A-PAGE) was determined from calibration curve for haemoglobine (Mr ~ 68 kDa; polypeptides: 52; 30 and 15.5 kDa, Serva Heidelberg, GE); lysozyme (Mr ~ 14.3 kDa, Sigma St. Luis, USA) and bovine serum albumin (Mr ~ 66.7 and 66.2 kDa, Imuna Šarišské Michaľany, SK). Electrophoresis was done in vertical unit (20

Table 1. Virulence analysis of some barley powdery mildew selected pathotypes

Pathogen	Genotype/*R*-gene			
	D/–	P02/*Ml-a3*	P04B/*Ml-a7*	P10/*Ml-a12*
RU-3	+	–	+	+
Sk-5-11	+	HR	HR	+

+ = compatible interaction; – = incompatible interaction
HR = hypersensitive interaction; *R* = resistance genes

Table 2. Virulence genes identified in two barley powdery mildew pathotypes

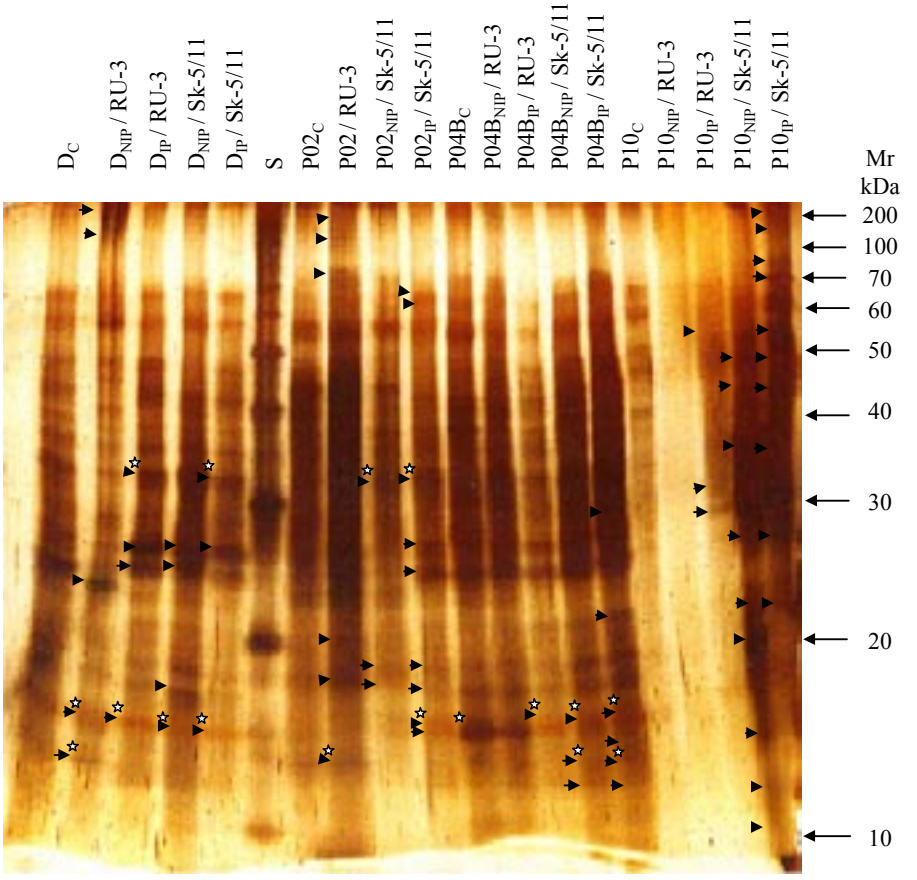
Pathogen	Virulence genes
RU-3	*av Ml-a3; v Ml-a7; v Ml-a9; v Ml-a12; v Ml-a13*
Sk-5-11	*av Ml-a3*; av Ml-a7*; v Ml-a12; v Ml-o*

* = hypersensitive interaction; *av* = avirulence gene; *v* = virulence gene

× 20 cm SCIE-PLAS, UK). Acid glucanases were detected according to PAN *et al*. (1991). Identification of chitinases after A-PAGE and SDS-PAGE was done according to TRUDEL and ASSELIN (1989).

RESULTS AND DISCUSSION

Accumulation of PR- and DR- proteins with hydrolytic function is ubiquitous reaction of mono- and dicotyledonous plants to pathogen infection and several abiotic stresses. During infection spectrum of hydrolytic enzymes changed quantitatively and qualitatively. In the period of powdery mildew sporulation, intracellular protein patterns (Figure 1) showed synthesis of large amount of PR and DR-proteins. These one were distributed, mainly by compatible and hypersensitive reactions (Table 2), along all patterns for intracellular extracts of the infected leaf. Differences in the PR-and DR-proteins compared to the adequate control lanes strongly depended on the genetic background both host and pathogens. The most intensive changes were detected in the interval 14–45 kDa by cv. Dvoran (without resistance genes from *Ml-a* locus) and isogenic line PO4B (resistance gene *Ml-a7*). Main quantitative and qualitative differences were for low molecular proteins and polypeptides. Biochemical analyses for acidic Glu (Figure 2) and Chi (Figure 1) showed that in the period pathogen sporulation (8th days after inoculation) were synthesized two groups of these hydrolytic enzymes. Nonspecific high molecular forms synthesized in the infected leaves had Mr < 200 kDa and were identified for all genetic combination between host and pathogen. Opposite situation was by low-molecular isoforms. Low molecular acidic Glu isozymes, Mr ~ 9–13 kDa, reflected both genetic differences of host genome and pathogen as well as distance from the infection site. Only non-infected and healthy plant of isogenic line P04B contained acidic Glu with Mr ~ 45 kDa. This isoform was synthesized in the infected dicotyledonous plants (SELA-BUURLAGE *et al*. 1993). The richest Glu pattern had isogenic line P10. Patterns for the infected and non-infected tissue contained three different Glu isoforms (Mr ~ 70–200 kDa) obviously non-synthesized by plants. This interval of Mr correspond rather to acidic Glu isoforms of bacterial and fungal pathogens (BIELY *et al*. 1985; CÔTÉ *et al*. 1989). This high molecular acidic Glu isoforms by plants detected only PAN *et al*. (1991) for tobacco leaves infected with *Perenospora tabacina*. Low molecular acidic Glu isoforms by barley isogenic lineP 10 reflected genetic differences between fungi.



C – control healthy plant of barley; NIP – fluid from uninfected barley leaf tissue; IP – fluid from infected barley leaf tissue; Arrows indicate PR- and DR- proteins; ✪ – indicate proteins with chitinase activity

Figure 1. SDS-PAGE protein patterns of different barley genotypes infected with two powdery mildew pathotypes in the period of pathogen sporulation (intracellular fluid)

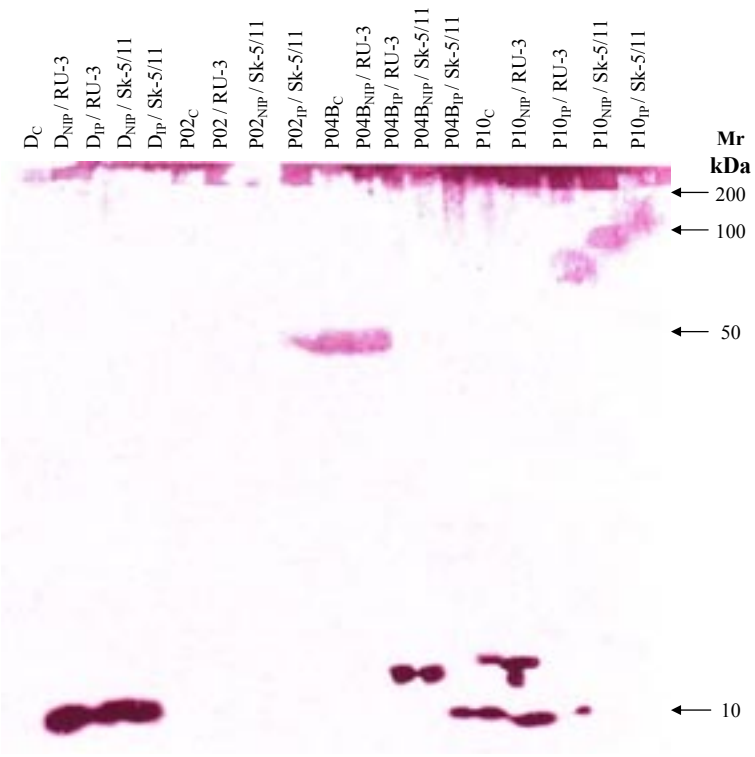


Figure 2. Intracellular glucanase zymogram of various barley genotypes infected with powdery mildew in the period of pathogen sporulation

C – control healthy plant of barley; NIP – fluid from uninfected barley leaf tissue; IP – fluid from infected barley leaf tissue

Low molecular chitinases, on the difference of their high molecular isoforms, reflected genetic differences between host and pathogen. Very weak presence or missing forms hydrolytic enzymes between 25–35 kDa, where is obviously localized most isoform of Glu and Chi indicated, that expression of corresponding genes was in this late phase of disease with high probability inhibited (HAM *et al*. 1997). Presence of abundant amount of low molecular isoforms by compatible interactions indicated on the possibility of the presence of some fungal enzymes with Glu and Chi activity. This assumption appeared to by true. Our morphological studies showed that the fungi haustorium covers ~ 30% volume of infected barley cells by compatible interaction

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