

Proteomic investigation of wheat intercellular washing fluid

Veronika Pósi¹, Krisztián Halász¹, Ákos Mesterházi², Lászlóné Csősz², Klára Manninger³, Éva Hunyadi-Gulyás⁴, Katalin Medzihradzky⁴, Tünde Juhász⁵, Noémi Lukács^{1*}

¹Department of Plant Physiology and Plant Biochemistry, Faculty of Horticulture, Corvinus University of Budapest, Budapest, Hungary, ²Cereal Research Non-Profit Company, Szeged, Hungary, ³Plant Protection Institute, Hungarian Academy of Sciences, Budapest, Hungary, ⁴Biological Research Center, Mass Spectrometry Facility, Hungarian Academy of Sciences, Szeged, Hungary, ⁵Biological Research Center, Institute of Enzymology, Hungarian Academy of Sciences, Budapest, Hungary

ABSTRACT The protein composition of wheat (*Triticum aestivum* L.) intercellular washing fluid was analysed to detect if there were changes in the protein pattern after fungal infection. The apoplast represents the first barrier to pathogens in the plant, and proteins which contribute to resistance against a given race of wheat leaf rust (*Puccinia recondita* f.sp. *triticii*) are expected to be found here. We compared the effect of leaf rust infection on two near-isogenic lines: the susceptible 'Thatcher' line and the corresponding near-isogenic Lr1-resistant line, which exhibits a hypersensitive response upon infection. Seven-day-old wheat seedlings were infected with Lr1 and the intercellular fluid was isolated at different times (1-7 days) after infection. Proteins were analysed by one- and two-dimensional gel electrophoresis followed by mass spectrometric analysis (MALDI-TOF) of selected protein species. Three apoplastic proteins, which appear earlier in the Lr1-resistant plants than in the 'Thatcher' line and which may therefore be involved in development of leaf rust resistance, were identified. One of the proteins is a glucan-endo-1,3- β -D-glycosidase (35413 Da), the second is a chitinase 1 enzyme (27077 Da) and the third one corresponds to the pathogenesis-related protein 1.1 (PR1.1, 17651 Da).

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Proteomics is the science of describing the protein composition of different organs, tissues, cell types, or even subcellular compartments and of identifying proteins associated with a particular physiological state. Proteomic methods, together with the rapidly growing wealth of genomic sequence databases provide an efficient tool for comparing the protein pattern of samples that differ in a defined way such as genetic background (e.g. pathogen resistance), treatment, etc. Proteins found to be specific for a given sample may serve as molecular markers for breeding purposes. To this end, proteomic approaches can be utilised to pinpoint potential target enzymes and proteins from pre-existing resistant lines that could be utilised to enhance the natural tolerance of agronomically favourable varieties of plants.

Selective breeding has produced numerous wheat varieties that show different types of resistance to leaf rust, and more than 40 races of the fungus have been identified. To facilitate understanding of leaf rust resistance a collection of nearly isogenic (NIL, >98% identical) wheat lines differing mainly in resistance to different leaf rust races was produced. Depending on the Lr (leaf rust resistance) genes present, the NIL lines show differences in the developmental stage of resistance manifestation and in the degree or appearance of symptoms. In addition, biochemical differences have also been reported in the literature,

such as the earlier appearance or constitutively higher levels of some proteins, *i.e.* antifungal, defence-related enzymes such as chitinases, β -1,3-glucanases etc. in some but not all resistant lines (Kemp et al. 1999; Angelova et al. 2001).

Materials and Methods

Plant materials

Seedlings of the wheat cv. 'Thatcher' and of the corresponding Lr1 line were grown in sterilised soil. The first leaves of seven-day-old seedlings were infected with a pathotype of leaf rust non-virulent on the Lr1 resistant line (7×10^5 spores/ml) in aqueous starch suspension. Samples were collected for 7 days after inoculation.

Isolation of intercellular washing fluid (ICF)

Isolation of ICF was carried out according to Rohringer et al. (1983), with some modifications: 20 mM Tris-HCl, pH 8.0 buffer containing 1 mM PMSF (Sigma) was used for infiltration (0.3 MPa, 30 min). ICF was isolated by centrifugation at 2000 rpm, 4°C, 20 min and was then adjusted to 8M urea concentration. It was stored frozen at -80°C until use.

Proteomic analysis

For one and two-dimensional SDS-PAGE, 12.5% (w/v) dis-

*Corresponding author. E-mail: noemi.lukacs@uni-corvinus.hu

continuous slab gels were prepared according to Laemmli (1970). Isoelectric focusing was carried out on IPG strips (12 cm, pH 3-10). After electrophoretic separation, proteins were stained either with CBB according to Rosenfeld (1992) and Neuhoff (1985) or by silver (Shevchenko 1996). Selected protein spots excised from the gels were reduced, alkylated with iodoacetamide and digested with trypsin. Samples were subjected to MALDI-TOF mass spectrometry. Proteins were identified by peptide mass fingerprinting and PSD sequencing.

Results and Discussion

Comparison of the ICF extracts of sensitive and resistant NIL lines 3 days after inoculation revealed several differences in the protein pattern. Three apoplastic proteins were identified which appear earlier (present exclusively or strongly overexpressed) after rust infection in the Lr1-resistant plants than in the 'Thatcher' line and which may therefore be involved in development of leaf rust resistance. One of the proteins is a glucan-endo-1,3- β -D-glycosidase (35413 Da, pI 8.8), the second is a chitinase 1 enzyme (27077 Da, pI 8.7) and the third one corresponds to the pathogenesis-related protein 1.1 (PR1.1, 17651 Da, pI 8.7). The identification of proteins is based on following evidence.

Identification of glucan-endo-1,3- β -D-glycosidase

The protein was strongly induced in the Lr1 resistant line and was detected in several gel systems. MALDI-TOF analysis of the tryptic peptides suggested that it probably represents a glucan-endo-1,3- β -D-glucosidase (also called glucanase), because several peptides having molecular masses characteristic for this enzyme were detected. The positively identified peptides covered 36-44% of the enzyme molecule. In addition, three of the peptides were sequenced and the sequences were found to be identical to the glucanase of *T. aestivum* cv. '75141' (ID 3757682), except for a conservative Ser \rightarrow Thr amino acid exchange at position 220 in Lr1. Two barley proteins (ID 3068599 and 21693553) also showed high similarity to the partial Lr1 sequences. The expected molecular mass of the Lr1 glucanase is also in good agreement with the experimental results. We found experimental evidence that the glucanase detected in ICF is indeed a secreted protein: PSD sequencing showed that the first peptide starts with an N-terminal isoleucine and not with a tryptic cleavage site, *i.e.* the 28 amino acid long signal peptide is cleaved from the glucanase in the endoplasmic reticulum leaving Ile at the N-terminus of the mature, secreted protein.

Identification of a chitinase 1 enzyme

Traces of the putative chitinase 1 were detected in the 'Thatch-

er' line, therefore the resistant and sensitive lines differ mainly in the expression level of this protein. The peptides identified cover 29% of the protein and two peptide sequences (21 amino acids in total) are identical to a *T. aestivum* chitinase 1 sequence (EMBL database ID 18146825). This family of chitinases is highly conserved in cereals and nearly identical sequences have also been found in barley (ID 563489), rye (ID 1240764) and rice (ID 37536556). Chitinases belonging to enzyme classes I-IV and characterised in the wheat varieties 'Chinese Spring', 'Sumai 3' and in 'Ning 7840' show much lower similarity to the Lr1 protein.

Identification of the pathogenesis-related protein 1.1 (PR-1.1)

Tryptic peptides covering 47% of wheat PR-1.1 proteins were detected by mass spectrometry and the amino acid sequences of two peptides were identical to those of PR-1.1 sequences found in the wheat cv. 'Kanzler' (ID: 3702663 and 14334165 in EMBL). Further homologues were found in other cultivars of wheat and in barley and rice. PR-1.1 identified in the ICF of the Lr1-resistant line is probably secreted, because it does not contain the signal peptide.

Taken together the results indicate that at least three proteins known to contribute to the antifungal response of plants are expressed earlier and/or at higher concentration in the apoplast of the Lr1-resistant line than in the corresponding sensitive 'Thatcher' line.

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