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ARTICLE

Isolation and characterization of EBR specific induced chitinases from tobacco (*Nicotiana tabacum*)

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ABSTRACT Early basal resistance (EBR) is a quick resistant response for pathogenic attack. Apoplastic proteins in plant intercellular space may play important role in triggering this response. Two chitinase enzyme –which's appearance is strongly connected to the early stage of this process - were isolated on HIC and further analysed. N-terminal aminoacid sequence were determined and their identification as IV class chitinases was proved. Our aim is to widen our knowledge about the expression, regulation of these enzymes. Also to analyse their role in the pathway leading from pathogen perception to resistance appearance.
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KEY WORDS
basal resistance
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Early basal resistance (EBR) is a quick, non-specific resistance response induced by saprophytic and pathogenic bacteria. It can be induced by general elicitors (conservative parts of bacteria). Activity of *hrp* (induction of HR and pathogenicity) genes of the bacterial pathogen (Somlyai et al. 1986) is required for induction of the hypersensitive reaction (HR, Klement and Goodman 1967) and EBR is able to inhibit the expression of bacterial *hrp* genes during the HR induction time (Bozsó et al 1999; Klement et al 1999). Previous studies carried out in our lab have shown that there are two chitinases whose presence and activity closely accompanies the EBR (Ott et al 2006). The role of these enzymes is still unclear in this context. They differ little in their amino acid sequence, size, isoelectric point, thus their separated analysis is challenging. Our first aim was to isolate and further characterize these extracellular chitinases. Because EBR is symptomless and relatively 'mild' form of resistance, its exploitation could improve self-defense of plants without losses in crop yield and quality.

Materials and Methods

Plant material: Experiments were carried out on 2.5-month-old tobacco plants (*Nicotiana tabacum* cv Samsun. EBR was induced with a suspension of heat-killed (15 min 72°C) avirulent *Pseudomonas syringae* pv *phaseolicola* (*P. phaseolicola)* - which is pathogenic on bean and incompatible in tobacco- ($4x10^8$ cell/ml), injected into the leaf intercellular space with a syringe. These freshly killed cells no longer induce the HR but carry active general EBR elicitors on their surface or in their cytoplasm. After injection, plants were kept at 27°C, because at this temperature EBR develops very quickly, and EBR specific chitinases reach a relatively high level 12 hours after injection. At this time point leaves were cut from the plant, and infiltrated with destilled water. This fluid (intercellular washing fluid = IWF), containing water-soluble apoplast proteins including inducable EBR specific chitinases, was extracted by centrifuging the leaf pieces at 600 g, at 4°C, for 10 min. Partial cleaning and prefractionation was carried out with the help of three phase partitioning (TPP). In TPP ammonium–sulphate and tert-butanol are used for separation of proteins by collecting and concentrating them into the middle layer or third phase of the system. This effective method preserves the activity of several enzymes and helps to get rid of other contaminants like polysaccharides, unwanted proteins and coloric agents. (Dennison et al. 1997) Chitinase activity test: One dimensional polyacrylamide gelelectrophoresis (1D-PAGE) separation in glycol-chitin containing acrylamide gel. After fluorescent staining protein bands showing chitinase activity appear in UV light like dark bands against the light background. Hydrophobic interaction chromatography (HIC) was carried out on a 1ml Phenyl FF low sub (GE Healthcare) column, the start buffer was 25mM Bis Tris pH 6,5; elution buffer: 1,5M $(NH_4)_2SO_4$.

Results and Discussion

The aim of our experiments was to extract EBR specific chitinases (Ott et al. 2006) in pure and active form in a relatively big amount to be able to examine their antimicrobial effect and to clearify their role in the pathway of induced resistance. The starting material was 400ml IWF gained from approx. 600 tobacco leaves injected with heat-killed bacterial cells. The protein content of IWF is very low, making the extrac-

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tion and analysation of these proteins tedious - including our target chitinases. Its original concentration is about 40 µg/ml IWF. The IWF proteins were concentrated 100 fold by ultrafiltration on a 10 kDa filter. A preliminary precipitation was carried out with different salt concentrations to determine the most appropriate parameters during HIC. The amount of precipitated active chitinase enzyme were followed by chitinase activity test. On the basis of that it can be concluded, that the major amount of chitinases were precipitated between 50-65% $(NH_4)_2SO_4$ concentration. Using the TPP method preliminary purification, prefractionation and further concentration of apoplastic chitinases was carried out. TPP eliminates impurities, leaving them in the aquous and in the organic phase. Proteins precipitated in the third phase (Szamos et al. 1998) were re-solubilised and separated on 1D-PAGE, which was then followed by chitinase assay. It can be concluded that our target chitinases retained enzymatic activity after the procedure. A constitutively expressed chitinase was selectively eliminated from the system, making easier the analysation of the two target chitinases (designated 215 and 250). This prefractionated sample was further fractionated by HIC. The 215 and 250 chitinases turned out to have a hydrophobicity difference that allowed to separate them into two different HIC fractions. Earlier separation attempts using isoelectric focusing, ion exchange chromatography or size exclusion chromatography had not resulted in satisfactory resolution, due to the physico-chemical similarity of the two enzymes. Although the resolving capacity of HIC has proved to be powerful, the efficiency of this fractionation method is still not optimal for our purposes, because too much chitinase activity was lost during HIC fractionation. The sequence of the last five N-terminal amino acid of the longer chitinase isoenzyme (250) was determined. Database searching revealed it to be identical with the N-terminal sequence of a class IV chitinase, the full gene sequence of which was determined by Shinya et al. (2007) and of which a partial sequence was cloned earlier by us (accession number: CAI54289). On the basis of the sequence similarity, affinity cromatography and at the cDNA level it could be proved that EBR induced chitinases lack a chitin binding domain in the N-terminal region.

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