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The insectivorous sundew (Drosera rotundifolia, L.) might be a novel source of PR genes for biotechnology

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The gene pool of insectivorous sundew, *Drosera rotundifolia* L., was studied to identify and analyse sequences encoding for pathogenesis-related (PR) proteins. The digested genomic DNA was in "inverted" Southern hybridisation probed to 19 clones for PR genes from different plant sources. From representatives of PR subgroups 1–5, 8 and 9, genes for glucanases (PR-2), chitinases (PR-3) and thaumatin-like proteins (PR-5) were hybridising. A PCR approach using degenerated primers was chosen to isolate sequences of sundew glucanase gene. Translation of a 500 bp long putative glucanase revealed similarity to catalytic domain of other glucanase amino acid sequences. Despite the peculiarity of this sequence, it contains all conserved amino acid residues important for catalysis. The sequence obtained in this study represents one of the first sequences encoding for nuclear genes in sundews reported, and brings the first evidence for presence of glucanases in sundew. The potential use of this sequence in biotechnology is considered as well.

Key words: glucanase, *Drosera rotundifolia* (L.), sundew, primary structure, inverted Southern hybridisation.

Introduction

Control of plant pests by the application of biological agents holds great promise as an alternative to the use of chemicals (Melchers & Stuiver, 2000). In spite of that it has been estimated that only 5 to 15% of the 250,000 to 500,000 existing species of higher plants have been surveyed for biological activity (Spjut, 1985). The revolution in biotechnology has generated even stronger interest towards identifying and isolating genes that encode for valuable bioac-

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tive compounds. Genes involved in plant defence against pathogens, so called pathogenesis-related (PR) genes (Van Loon & Van Stieren, 1999) comprising 17 subgroups (PR1-PR17), have been the most extensively investigated in efforts to produce various pest resistant plants (Strittmater & Wegener, 1993). Their successful use, however, has been shown to have some limitations. For example, only PR proteins of specific groups have been shown to be toxic to tested pathogens *in vitro* (Sela-Buurlage et al., 1993). In addition, plant pathogens are often tolerant to (toxic) metabolites produced by their host, but sensitive to structurally related metabolites from other non-host plants (Neuhaus et al., 1991; Joosten et al., 1995; Susi et al., 1995). Plant species that are genetically distant from crops are therefore gaining on interest.

In this work the sundew (*Drosera rotundifolia* L.), a representative of insectivorous plants, was searched for presence of PR genes. Until now chitinases of glycoside hydrolase family GH-19 (Henrissat, 1991), belonging to the group of PR-3, have been detected in digestive fluid of *Nephentes* and few *Droseraceae* (Amagase et. al., 1972; Robins & Juniper, 1980). However, except for a few genes encoding chitinases (unpublished sequences with GenBank Accession No. AY618884 and AY643484), no further sequence data on presence of PR genes in insectivorous plants are available.

In this paper we paid attention to endo-1,3-β-glucanases (EC 3.2.1.39), the other member of PR group (PR-2). These plant enzymes belong, in the sequence-based classification of glycoside hydrolases, to family GH-17 (Henrissat, 1991). They hydrolyse polysaccharides found in the plant and fungal cell walls, thus functioning in plant development and in defence of the plant against fungal diseases (Neale et al., 1990; McCollum et al., 1997; Caruso et al., 1999). Especially in combination with chitinases, glucanases showed fungitoxic effects *in vitro* and *in planta* that rendered them very interesting for gene transfer technology (Sela-Buurlage et al., 1993; Jongedijk et.al., 1995).

Exploiting the conservative nature of specific PR genes (Meins et al., 1993), a genomic sequence encoding for a putative glucanase gene fragment was isolated using degenerated primers. This sequence, representing the first glucanase fragment obtained from an insectivorous plant, was analysed and compared with homologous sequences from another plant species.

Material and methods

Plant material

Plants of *Drosera rotundifolia* L. (*Droseraceae*, genus *Drosera*) were grown on solidified MS medium (Murashige & Skoog, 1962) pH 4.5, containing 2% sucrose without any growth regulator. Plants were maintained at a day-night regime of 24 °C ± 2 °C, under a 16-h photoperiod using cool white fluorescent lights with a photo flux of 80 $\mu M \cdot m^{-2} \cdot s^{-1}$ (400–700 nm).

Southern hybridisations

DNA manipulations were performed using the standard molecular biology techniques (Sambrook et al., 1989). For Southern hybridisation, 10 μg of total DNA (Békésiová et al., 1999) was digested with the restriction enzyme *Hind* III or *Bam*H I, separated on a 0.9% (w/v) agarose gel and blotted by vacuum-blotter (VacuGene XL and VacuGene Pump, Pharmacia LKB) onto a Hybond N^+ membrane (Amersham). The appropriate DNA fragment used as a probe was radioactively labelled with ^{32}P (Amersham Megaprime DNA labelling Kit). The hybridisation was performed in hybridisation solution containing 10% (w/v) dextran sulfate, 1% (w/v) SDS, 1 $mol \cdot L^{-1}$ NaCl and 100 $\mu g \cdot mL^{-1}$ of salmon sperm DNA at 65 °C. Blots were washed in 2 × SSC, 1% (w/v) SDS at hybridisation temperature for 15–20 min, and 1 × SSC, 1% (w/v) SDS for 2–5 min. Hybridisation signals were visualized by autoradiography using a BAS2000 PhosphorImager (Fuji).

For inverted Southern hybridisation, a set of PR gene fragments (Table 1) was restricted from plasmids according to provider's instructions. Isolated fragments were separated on 1% agarose gel, blotted and hybridised at 55 °C for 72 h as described. As for a probe, 10 μg of sundew genomic DNA was digested with *Eco* RI. The digest was precipitated with 1/10 volume of 3 $mol \cdot L^{-1}$ sodium acetate (pH 5.2) and 2.5 volume of 96% ethanol (v/v) at −70 °C, for 20 minutes. After centrifugation (14,000*g*, 10 min at 4 °C) the DNA was washed and dried under vacuum. The pellet was re-suspended in 15 μL water and radioactively labelled as described.

Isolation of a glucanase sequence from sundew

A glucanase gene sequence was isolated from sundew by PCR on genomic DNA. Degenerated primers were designed based on conservative domains RYIAVGNE (forward), SESGWPS and ETYIFAMF (reverse) of nine genes from distantly related plant species (citrus, tobacco, rubber tree, maize, barley and rice) (GenBank accession numbers AJ000081, X54456, U22147, M95407, M96939, AF030167, respectively). The primers, including the restriction sites (underlined) for *Kpn* I (forward) and *Bam*H I (reverse), were:
GluFor [5′- ATGGATCCTC(A/C)(A/G)ITA(T/C)-AT(A/C/T)GCIGTIGGIAA(C/T)G]
GluRev1 [5′- ATGGTACCG(T/A)(C/A)GGCCAIC-C(G/A)CT(C/T)TCIG]
GluRev2 [5′- ATGGTACCAACA(T/G)(G/A)GC-(G/A)(A/G)AIAIGT(A/T)IGTCTC]

Table 1. DNA clones encoding for PR proteins that were used in "inverted" Southern hybridisation.

Gene[a]	Source	PR class	Function	GeneBank Acc. No.
PR1-a	tobacco	1	?	X12737
PR1-b	tobacco	1	?	X14065
PR2	tobacco	2, subclass II	glucanase	M60460
PR3	tobacco	3, subclass II	chitinase	M23868
PR4	tobacco	4	?	X60281
PR5	tobacco	5	thaumatin like protein	JH0230
CHIII-a	tobacco	3, subclass III	chitinase	Z11563
CHIII-b	tobacco	3, subclass III	chitinase	Z11564
acidic peroxidase	tobacco	9	peroxidase	U15933
CHII	rice	3, subclass II	chitinase	L40336
RC24	rice	3, subclass I	chitinase	X87109
CSC121	citrus	3, subclass II	chitinase	Z70032
Gn1*	tobacco	2, subclass I	glucanase	X07280
G101	alfalfa	2, subclass I	glucanase	U27179
G46	potato	2, subclass I	glucanase	U01901
ChtB3	potato	3, subclass I	chitinase	U02607
CHpBS	cucumber	8	chitinase	M24365
WIR232	wheat	5	thaumatin like protein	X58394
PR-Q	tobacco	2, subclass III	glucanase	X54456

[a] a – acidic; b – basic; * – genomic clone.

The PCR profile was: 1 cycle of 94 °C for 1 min; 35 cycles of 94 °C for 1 min, 54 °C for 1 min, 72 °C for 2 min; 1 cycle of 72 °C for 7 min.

The putative gene fragment was cloned into pGem®-T Vector System II (Promega). The insert was sequenced in both directions using M13 primers on a Perkin Elmer 377 DNA Sequencer using a BigDye™ Terminator Cycle Sequencing Ready Reaction Kit (Promega). The sequence was subjected to BLAST analyses (Altschul et al., 1997). Sequence alignments with homologous genes obtained from GenBank were performed using CLUSTAL W (Thompson et al., 1994).

Results and discussion

Pathogenesis-related proteins are ubiquitous in plant kingdom. Many of them have been shown to contribute to plant defence against invading pathogens that rendered them very attractive for biotechnology as an alternative approach to fungicide use on food crops (Strittmatter & Wegener, 1993; Shah, 1997). Hundreds of genes encoding for different PR members from various plant species have been identified and cloned, and there is an increasing interest for isolating furthers from novel, unusual (non-crop) sources. In the frame of this concept the insectivorous sundew was screened for PR sequences. The broad scale of their homologues in this plant was narrowed by hybridisation of PR genes from different plant sources to the sundew genomic DNA. Out of 19 fragments comprising members of PR groups 1–5, 8, 9 (Fig. 1) mostly chitinases were hybridising. This is probably attributed to relative high interspecific conservation of chitinases (53.4%) (Meins et al., 1993). The strongest hybridisation revealed chitinase clones encoding for a tobacco class II chitinase PR-3 (Payne et al., 1990) and potato chitinase of class I ChtB3 (Beerhues & Kombrink, 1994). The most similar glucanase (interspecific conservation 42.9%) (Meins et al., 1993) appeared to be the tobacco glucanase of class III PR-Q' (Payne & Ryals, 1990). The tobacco PR-5 gene (encodes for a thaumatin-like protein) (Payne et al., 1988) revealed potentially higher similarity to sundew sequences as well. Weaker hybridisation signals were obtained for chitinases CHII (Kim et al., 1998), RC24 (Xu et al., 1996) both from rice and CSC121 (Nairn et al., 1997) from citrus, glucanases G101 (Maher et al., 1993) from alfalfa and a tobacco genomic clone Gn1 (De Loose et al., 1988), and for clone encoding the wheat thaumatin-like protein WIR232 (Rebmann et al., 1991).

The modified method of DNA-DNA hybridisation that we used (inverted Southern hybridisation) has an advantage of testing several DNA fragments in one experiment for their capability to hybridise to the given genomic DNA. Prolonged time of hybridisation (here 72 h) and low hybridisation and washing temperatures were chosen to favour the probe to find and bind target sequence.

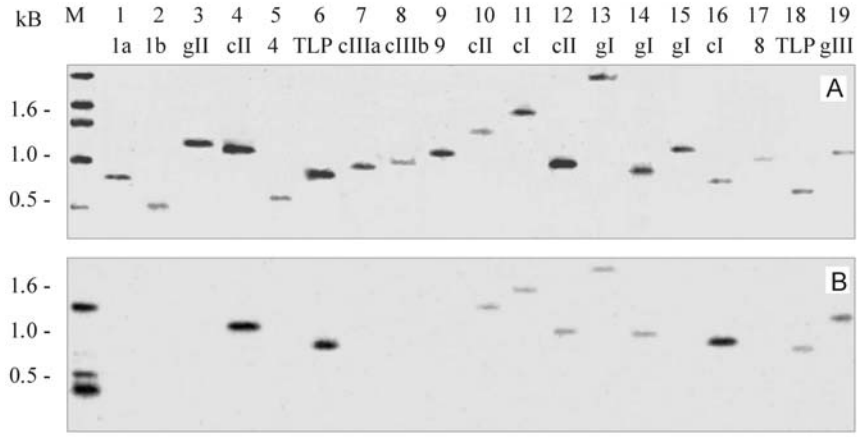

Fig. 1. Inverted Southern hybridisation of sundew DNA to PR genes. (**A**) A set of different fragments encoding for PR genes (1–19) was separated on 1% agarose gel and stained with Ethidium bromide. For clarity, the greyscale of gel was inverted. (**B**) Autoradiograph of the hybridised PR genes (1–19). As a probe, 10 μg of sundew genomic DNA, digested with *Eco*R I and radioactively labelled with ^{32}P was used. M – 1 kb DNA ladder (Life Technology). Lanes: (*1*) – PR-1a (X12737), (*2*) – PR-1b (X14065), (*3*) – PR-2 (M60460), (*4*) – PR-3 (M29868), (*5*) – PR-4 (X60281), (*6*) – PR-5 (JH0230), (*7*) – CHIIIa (Z11563), (*8*) – CHIIIb (Z11564), (*9*) – PR-9 (acidic peroxidase, U15933), (*10*) – CHII (L40336), (*11*) – RC24 (X87109), (*12*) – CSC121 (Z70032), (*13*) – genomic clone Gn1 (X07280), (*14*) – G101 (U27179), (*15*) – G46 (U01901), (*16*) – ChtB3 (U02607), (*17*) – CHpBS (M24365), (*18*) – WIR232 (X58394), (*19*) – PR-Q' (X54456). Abbreviations of PR groups and their subclasses are viewed: (*1a*) – acidic PR-1, (*1b*) – basic PR-1, (*gI – gIII*) – glucanases (PR-2) of subclasses I–III, (*cI – cIII*) – chitinases (PR-3) of subclasses I–III, (*cIIIa*) – acidic chitinase of class III, (*cIIIb*) – basic chitinase of class III, (*4*) – PR-4, (*8*) – PR-8, (*9*) –acidic peroxidase (PR-9), (*TLP*) – gene for thaumatin-like protein (PR-5).

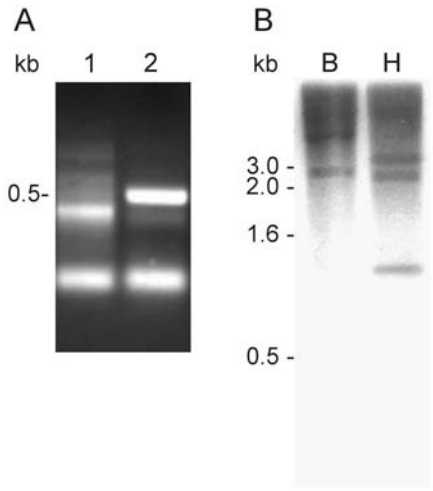

Fig. 2. Identification of glucanase gene sequences from sundew. (**A**) PCR using primers for glucanases. The primer combination DrGluFor/Rev1 yielded a smearing product (1), and the DrGluFor/Rev2 a strict $\sim$ 500 bp fragment (2). (**B**) Autoradiograph of sundew genomic DNA digested with*Bam*H I (B) or *Hind* III (H). The blot was hybridised with ^{32}P labelled glucanase fragment DrGlu1 as a probe.

These factors might be important especially in case of very large genomic DNA used as a probe, or when low copy number of the studied gene is present in it. Too long or too short fragments in the DNA mixture of probe, as well as unefficient labelling can negatively influence the final result as well. The proper restriction enzyme for cutting genomic DNA must be considered (e.g. frequent or rare cutter, performing complete digestion, specific base composition of the genome depending on the plant source). Some of detected homologues (chitinase clones ChtB3, PR-3 and the glucanase PR-Q') have been successfully probed to sundew genomic DNA in "classical" Southern hybridisation (autoradiographs not shown). The approach of "inverted" Southern hybridisation enables then in relatively shorter time and cheaper way to select specific genes (gene groups) that could be used as a starting point for further research on a poorly studied genome, e. g., as probes to screen libraries or to design PCR primers.

Degenerated primers for glucanases were designed on the base of conservative domains of nine glucanase genes from the GenBank, while highly variable positions were replaced by corresponding nucleotides in the sequence of PR-Q'.

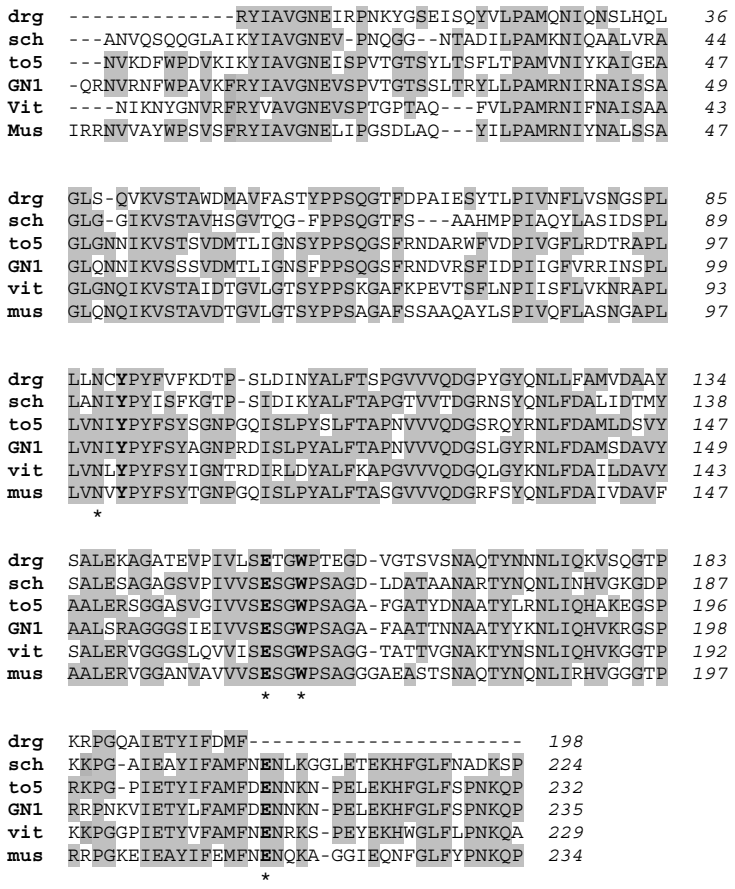

Fig. 3. Alignment of deduced amino acid sequence of the sundew putative glucanase fragment DrGlu1 with other plant glucanases. Amino acids are shown in one-letter code. The residues that are similar in at least three sequences are shaded. The amino acids, that are conserved in all genes and are important for enzyme catalysis, are signified with bold letters and asterisk. (**drg**) – sundew glucanase (AY622820), (**sch**) – glucanase Glu1 from *Schedonorus pratensis* (CAC40810), (**to5**) – tobacco glucanase (CAA37669), (**GN1**) – tobacco glucanase translated genomic clone Gn1 (CAA01265), (**vit**) – grapevine glucanase (CAB91554), (**mus**) – banana glucanase (AAF08679).

The PCR amplification yielded a smearing product using primer combination GluFor/Rev1, and one strict band of ∼500 bp using primer combination GluFor/Rev2 (Fig. 2A). As none of these amplicons did hybridise after blotting and probing to the glucanase clone PR-Q' (autoradiograph not shown), the ∼500 bp long fragment, being of expected size, was cloned and sequenced. BLAST search (Altschul et al., 1997) revealed that a chain of 32 nucleotides of DrGlu1 was only identical with a sequence of β-1,3-glucanase cDNA At3g57260 from *Arabidopsis thaliana*. This implicates to very low degree of sequence similarity to other genes at nucleotide level. The translated sequence, however, shared significant similarity to β-1,3-glucanases from different plant sources (Fig. 3). The most similar primary structure of banana glucanase (AAF08679) revealed more than 54% identities and 71% positives in the alignment. For comparison, Meins et al. (1993) reported 42.9% inter-specific conservation in a weighted average of 18 representative glucanase amino acid sequences from 6 plant species. Such contrast between results of BLAST searches at nucleotide and translated sequence (protein) levels might be a consequence of frequently occurring synonymous codons, and can also be found at some other genes from insectivorous plants. For example, a 1056 bp long sequence from *Nepenthes* (AY618884) is after translation (except to

the first 300 bp) significantly similar to chitinases from other plant species. At nucleotide level, however, two to four areas of <100 bp share similarity with chitinases from other plant species. Further examples can be the chitinase gene *Chit1* from *Drosera spathulata* (AY643483), chitinase gene fragment from *Dioneaea muscipula* (*Droseraceae*) (AY643484) and gene *DtatpG-2* (AB112339) for a hypothetical protein ATP synthase subunit from *Drosera tokaiensis*. Such a contradiction cannot be observed in case of cytosolic glutamine synthase gene *DtCGS-1* (AB112466) from *Drosera tokaiensis* and some *rbcL* genes. The few available sequence data of nuclear genes from insectivorous plants are not sufficient to make any deduction concerning this phenomenon.

Recent discoveries of divergent new genes in glycoside hydrolase family GH-17 (Romero et al., 1998) have added to the complexity of this gene family. In spite of that there is a set of conserved sequence regions that are shared by all members of the gene family (Hird et al., 1993) and may be important for catalysis. Out of five positions reported (Meins et al., 1993), the corresponding three ones can also be found in the sundew sequence (Y181, E244 and W248) (Fig. 3). The glucanase gene region under discussion contains usually no intervening sequences and is highly conservative.

The origin of obtained DrGlu1 sequence was verified in Southern hybridisation using it as probe (Fig. 2B). Sequence comparisons and Southern blot analyses suggest that glucanases in sundew are encoded by small gene families with the potential of 3 members. By analogy to other plant and animal genes, it is likely that these families arise after the duplication of a single gene by recombination and gene conversion (Maeda & Smithies, 1986).

Further research on DrGlu1 will be focused on obtaining the entire gene sequence and expression studies. The peculiarity of the isolated partial sequence of DrGlu1 might be reflected in the protein with specific properties that in turn might sketch new ways of its exploitation in biotechnology. Research on pathogenesis-related proteins and their genes in this special plant species can in addition contribute to understanding of the fundamental mechanisms of their action.

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Plasmids containing cDNAs for tobacco PR proteins (PR-1a, PR-1b, PR2, PR-3, PR-4, PR-5, CHIII-a, CHIII-b, acidic peroxidase and PR-Q') and a cucumber chitinase of class III (CHpBS) were kindly provided by Dr. Lawton, NOVARTIS Crop Protection, Inc., Switzerland. Plasmids containing cDNAs for potato chitinase ChtB3 and glucanase G46 were kindly provided by Dr. Kombrink, Max Planck Institut für Zuchtungsforschung, Köln, Germany. The glucanase cDNA clone G101 was kindly provided by Dr. Dixon, The Samuel Roberts Noble Foundation, Inc. Oklahoma, USA. The glucanase genomic clone Gn1 was obtained from Dr. Van Montagu, Gent, Belgium. The cDNA clone WIR 232 was obtained from Dr. Dudler, Institute for Plant Biology, University of Zürich, Switzerland. This work was supported by INCO-Copernicus project IC15-CT96-0921, grant No. 2/1154/21 from the Slovak Grant Agency VEGA, and APVT grant No. 51-00-56-02.

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