



## A birch gene family encoding pollen allergens and pathogenesis-related proteins

Ines Swoboda<sup>a</sup>, Otto Scheiner<sup>b</sup>, Dietrich Kraft<sup>b</sup>, Michael Breitenbach<sup>c</sup>,  
Erwin Heberle-Bors<sup>a</sup>, Oscar Vicente<sup>a,\*</sup>

<sup>a</sup> Institute of Microbiology and Genetics, University of Vienna, Vienna Biocenter, Dr. Bohr-Gasse 9, A-1030 Vienna, Austria

<sup>b</sup> Institute of General and Experimental Pathology, University of Vienna, General Hospital, Währingergürtel 18-20, A-1090 Vienna, Austria

<sup>c</sup> Institute of Genetics, University of Salzburg, Hellbrunnerstr. 34, A-5020 Salzburg, Austria

Received 15 December 1993; revised 29 April 1994

### Abstract

*Bet v* 1, the major pollen allergen of birch (*Betula verrucosa*), shows high sequence homology to a family of pathogenesis-related (PR) proteins that have recently been identified in several other plant species. We have used a pollen *Bet v* 1 cDNA clone and anti-*Bet v* 1 antibodies as probes to study the expression of *Bet v* 1 genes in birch cell suspension cultures under different experimental conditions. Induction of *Bet v* 1-related proteins was detected in immunoblots of cell extracts upon co-cultivation with microbial pathogens. Northern analysis revealed the rapid induction of *Bet v* 1 transcripts in the presence of bacteria and fungi, but not by stress treatments (heat shock, metal ions) or by chemical elicitors. RNase protection experiments showed that the pathogen-inducible RNAs did not correspond to the pollen cDNA clone but most likely to the products of transcription of other members of the *Bet v* 1 gene family, sharing high sequence homology with the pollen-specific gene within the 5'-half of the coding region. We conclude that the *Bet v* 1 gene family of pollen allergens includes a subset of defense-related genes that are transcriptionally activated in the presence of microbial pathogens.

**Keywords:** Allergy; *Bet v* 1; Microbial pathogen; Pollen; Pathogenesis-related protein; (*Betula*)

### 1. Introduction

Pathogenesis-related (PR) proteins are a heterogeneous group of host-encoded, low molecular weight proteins that are induced in plant tissues by infection with pathogens (fungi, bacteria, viruses or viroids) or by treatment with biotic or chemical elicitors. The synthesis of specific pathogen-inducible proteins can also be activated under different stress conditions, including heat shock, wounding, anaerobiosis, hormonal shock or treatment with a large number of chemicals and metal ions (see [1-4] for reviews). Two groups of PR-proteins have been identified as chitinases and 1,3- $\beta$ -glucanases [5-8]. Since these hydrolytic enzymes can digest the main component of the cell walls of fungi and bacteria, it is likely that they have a direct anti-microbial effect in the defense reaction of the plant against pathogens.

For most PR proteins, however, the biological activity is still unknown.

Recently, a new class of ubiquitous, highly conserved defense-related proteins has been described. Members of this family of PR-proteins are transcriptionally activated upon infection with bacterial or fungal pathogens, or by treatment with fungal elicitors, in bean [9] and parsley [10,11] suspension cultures, in pea pods [12,13] and in potato tuber disks [14]. Homologous genes are induced by wounding in asparagus [15] and under different stress conditions (including also wounding and treatment with fungal elicitor) in soybean [16]. Similarities in amino acid sequence, protein size (about 17 kDa) and total charge clearly indicate a common ancestry and most probably an identical or related, still unknown function for these proteins.

Interestingly, an allergenic birch protein seems also to belong to this class of defense-related proteins. *Bet v* 1, a polypeptide of 17 kDa, is the major birch pollen allergen, responsible for IgE binding in more than 95%

\*Corresponding author. Fax: +43 1 7986224.

of patients allergic to birch pollen. Several *Bet v* 1 isoforms, of the same molecular weight, are constitutively expressed at high levels in mature pollen and efficiently released upon pollen hydration, so that they constitute the most abundant protein species in aqueous pollen washes. A *Bet v* 1 cDNA clone was isolated from a pollen expression library by immuno screening with a patient's serum [17]. The deduced amino acid sequence showed high homology to the above-mentioned group of PR-proteins. A putative functional role of *Bet v* 1 in pathogen resistance of pollen has been proposed, based on this homology. However, no data on pathogen-related expression of *Bet v* 1 exist.

We have set up a birch cell suspension culture as a convenient experimental system to study the expression of *Bet v* 1 genes, at the RNA and protein levels. Our aim was to investigate whether the genes expressed constitutively in pollen, or other members of the *Bet v* 1 gene family, are transcriptionally activated by microbial pathogens.

## 2. Materials and methods

### 2.1. Plant material

Surface-sterilized seeds of birch (*Betula verrucosa*) were germinated on MS [18] agar plates at 25°C. Leaves from 1-week-old seedlings were used to initiate a callus culture on plates containing UM6 medium (MS macro and micro minerals, 0.1 mM Fe-EDTA, 3% sucrose, 1% MS vitamins, 0.5 mg l<sup>-1</sup> kinetin, 0.5 mg l<sup>-1</sup> dichlorophenoxyacetic acid and 25 mg l<sup>-1</sup>  $\alpha$ -naphthaleneacetic acid, pH 5.8) solidified with 1% agar. Suspension cultures were prepared from approx. 1 g of callus material, disaggregated and suspended in liquid UM6 medium. The cultures were maintained in Erlenmeyer flasks on rotary shakers at 28°C in the dark, and were subcultured once per week by diluting a sample 10-fold into fresh medium.

### 2.2. Cell treatments

Cultures in the rapid phase of growth, approx. 3 days after subculturing, were used in the experiments. Putative elicitors (arachidonic acid, salicylic acid) and ionic salts were dissolved in water, filter-sterilized and added to the cultures to yield final concentrations as indicated in the legends. The plant pathogens *Pseudomonas syringae*, *Xanthomonas campestris*, *Erwinia carotovora* and *Colletotrichum lindemuthianum* were obtained from the 'German Collection of Microorganisms and Cell Cultures' (Braunschweig, Germany) and *Fusarium solani* and *Taphrina betulina* from the 'Centraalbureau voor Schimmelcultures' (Baarn, The Netherlands). Fungi and bacteria were grown in agar

plates as recommended by the suppliers. Pathogen cell material was harvested with a sterile spatula and resuspended in sterile water to a concentration of 1 A<sub>600</sub> unit per ml. Aliquots of 50 ml of birch cells were supplemented with 1 ml of these pathogen suspensions and cultured at 28°C for the times indicated in the legends. Heat-shock treatments were performed at 42°C for 3 h.

### 2.3. Protein extracts and immunoblotting

1 g of birch pollen, obtained from Allergon AB (Engelholm, Sweden) was suspended in 20 ml of extraction buffer E (17.5 mM potassium phosphate buffer (pH 7.0), 2 mM EDTA, 10 mM DIECA) and gently shaken overnight at 4°C. After centrifugation (10000 rpm, 5 min at 4°C in a Sorvall ss34 rotor), the supernatant was concentrated by ammonium sulfate precipitation (80% saturation) and the pellet was dissolved and dialyzed against buffer D (17.5 mM potassium phosphate buffer, pH 7.0); the final sample, referred to as 'pollen wash', was frozen and stored in aliquots at -80°C until used. Cultured birch cells were collected by centrifugation, and ground in a mortar with buffer E supplemented with 100 mM NaCl and a mixture of proteinase inhibitors (0.1 mM PMSF, 1 mM benzamide, 2  $\mu$ g ml<sup>-1</sup> aprotinin, 2  $\mu$ g ml<sup>-1</sup> leupeptin, 100  $\mu$ g ml<sup>-1</sup> bacitracin, 1  $\mu$ g ml<sup>-1</sup> antipain). The homogenate was centrifuged at 15000 rpm for 20 min at 4°C in a ss34 rotor, and the supernatant, after ammonium sulfate precipitation, was extensively dialyzed against buffer D.

Polypeptides in pollen washes and cell extracts were separated in 15% PA/SDS gels [19] and transferred to nitrocellulose membranes by semi-dry blotting (Semi-Phor TE70 transfer unit, Hoeffer). The membranes were incubated either with the monoclonal antibody BP1, prepared against recombinant *Bet v* 1 protein [20], or with a pool of antisera from allergic patients; *Bet v* 1 proteins were then detected with an anti-mouse antibody coupled to alkaline phosphatase and colorimetric substrates (BCIP and NBT), or with <sup>125</sup>I-labelled anti-human IgE (Phadebas RAST radioimmunoassay system, Pharmacia) followed by autoradiography.

Protein concentration in pollen washes and cell extracts was measured by the method of Bradford [21] using the Bio-Rad reagent and BSA as standard.

### 2.4. Plasmids constructs

Standard procedures [22] were used to prepare the following constructs. (i) The whole *Bet v* 1 cDNA, originally cloned in the *Hind*III/*Eco*RI sites of *Bluscript* SK+ [17] was cut out from the vector with *Cla*I and *Sma*I and recloned in the *Cla*I/*Sma*I sites of pGEM7Zf(+) (Promega) generating the plasmid

pG7BetvIA. (ii) The *Stu*I/*Bam*HI fragment of *Bet v* 1 cDNA (bases 129–399) was cloned in the *Bam*HI/*Hinc*II sites of pGEM2 (Promega) giving rise to plasmid pG2BetvI. (iii) The *Bam*HI/*Afl*III fragment of the cDNA clone (bases 399–572) was cloned in pGEM7Zf(+) as follows: first, the *Bam*HI/*Eco*RI fragment of *Bet v* 1 cDNA was cloned in *Eco*RI/*Bam*HI-digested pGEM7Zf(+); the fragment obtained by digestion of this construct with *Afl*III, followed by filling-in with T4 DNA polymerase and digestion with *Bam*HI was finally cloned in the *Bam*HI/*Sma*I sites of pGEM7Zf(+), generating the plasmid pG7BetvIB. After enzymatic digestions, vectors and inserts were purified by low-melting-point agarose gel electrophoresis, and ligations were carried out directly in the melted gel slabs.

### 2.5. Synthesis of nucleic acid probes

<sup>32</sup>P-labelled DNA probes were prepared from the 270 bp *Bam*HI/*Hind*III fragment of plasmid pG2BetvI. The DNA was gel-purified and labelled by random oligonucleotide priming [22].

<sup>32</sup>P-labelled anti-sense RNA probes were synthesized by in vitro transcription [23] of the plasmids pG7BetvIA, linearized with *Cla*I (probe 1 in Fig. 3A), pG2BetvI, linearized with *Hind*III (probe 2) or pG7BetvIB, linearized with *Sac*I (probe 3), with T7 (probes 1 and 3) or SP6 (probe 2) RNA polymerases. Non-radioactive sense-RNAs corresponding to probes 1 and 2 were transcribed in vitro from the plasmid pG7BetvIA (linearized with *Sma*I), using the SP6 promoter, and from pG2BetvI (linearized with *Bam*HI) with T7 RNA polymerase, respectively. Full-length anti-sense RNA probes used in RNase protection assays were purified by electrophoresis in 6% PA/7 M urea gels [24].

### 2.6. RNA preparation

Total RNA was prepared from birch pollen, leaves and cell suspension cultures, and from bacteria as described by Chomczynski and Sacchi [25] including, as a final purification step, extraction with chloroform/n-butanol (4:1), followed by ethanol precipitation.

### 2.7. Northern blot analysis

RNA was separated by electrophoresis in 1.2% formaldehyde agarose gels [26] and transferred to nylon membranes (Hybond-N, Amersham) by capillary blotting in 10 × SSC overnight at room temperature. Filters were hybridized with <sup>32</sup>P-labelled probes, either DNA or anti-sense RNA, at 42°C for 20 to 24 h. In the case of DNA probes, prehybridization (1–4 h) and

hybridization were carried out in 25 mM potassium phosphate buffer (pH 7.4), 5 × SSC, 5 × Denhardt's solution, 50% formamide and 100  $\mu$ g ml<sup>-1</sup> sonicated salmon sperm DNA. Washings were performed twice in 2 × SSC, 0.1% SDS at room temperature for 20 min, and once in 0.1 × SSC, at 55°C for 30 min. In the case of anti-sense RNA probes, the prehybridization solution contained 50 mM sodium phosphate buffer (pH 6.5), 5 × SSC, 10 × Denhardt's solution, 50% formamide, 0.1% SDS and 250  $\mu$ g ml<sup>-1</sup> salmon sperm DNA; for hybridization, this solution was supplemented with 10% dextran sulfate. After washing as indicated for DNA probes, the membrane was incubated with RNase A (10  $\mu$ g ml<sup>-1</sup> in 10 mM Tris-HCl (pH 7.5), 300 mM NaCl, 1 mM EDTA) for 15 min at 37°C, and then washed in the same buffer without RNase A for 20 min at 37°C.

### 2.8. RNase protection assays

RNase A/T1 protection assays were carried out essentially following the procedure originally described by Myers et al. [27], as modified in [24]. <sup>32</sup>P-labelled, gel-purified anti-sense RNA probes (15000–20000 Cerenkov cpm) were hybridized to total RNA (10  $\mu$ g) prepared from birch pollen or cell suspension cultures, in 10  $\mu$ l of 40 mM Pipes (pH 6.7), 0.4 M NaCl, 1 mM EDTA, 80% formamide, for 12 to 17 h at 45°C. The reaction was stopped by addition of 100  $\mu$ l of RNase digestion buffer (10 mM Tris-HCl (pH 7.5), 200 mM NaCl, 100 mM LiCl, 1 mM EDTA) containing 4  $\mu$ g ml<sup>-1</sup> RNase A and 0.2  $\mu$ g ml<sup>-1</sup> RNase T1, and the sample was incubated at 26°C for 40 min. After proteinase K treatment, phenol/chloroform extraction and ethanol precipitation, non-digested fragments of the radioactive probe were analyzed by electrophoresis in 6% polyacrylamide/7 M urea gels followed by autoradiography in Kodak AR X-Omat films.

## 3. Results

### 3.1. Induction of *Bet v* 1-related proteins in birch cell suspension cultures

A monoclonal antibody, BIP 1, prepared against recombinant *Bet v* 1 [20] was used to detect *Bet v* 1 proteins in Western blots of birch cell extracts (Fig. 1). As a positive control, a birch pollen wash was also loaded on the gel (lane 1). In control cultures the antibody detected low levels of a protein of the same molecular mass, 17 kDa, as pollen *Bet v* 1 (lane 2). The intensity of this band clearly increased upon co-cultivation of the cells with bacteria or fungi (lanes 3 to 8). In addition, a second polypeptide of ca. 18 kDa, cross-reacting with BIP 1 and induced in the presence of

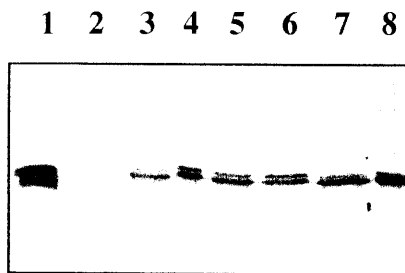


Fig. 1. Immunoblot of protein extracts from birch cells cultured in the presence of different bacteria and fungi. Aliquots of a suspension culture were incubated for 8 h without further additions (lane 2) or supplemented with *P. syringae* (lane 3), *F. solani* (lane 4), *E. carotovora* (lane 5), *X. campestris* (lane 6), *T. betulina* (lane 7) or *E. coli* (lane 8); all these samples contained 5 µg protein. As a positive control, a birch pollen wash (0.7 µg protein) was loaded in lane 1. The blot was developed with the anti-Bet v 1 monoclonal antibody BIP 1.

microbes, was reproducibly detected in the immunoblots. The relative intensity of these two bands was variable in different experiments and low levels of the 18 kDa protein were sometimes detected also in control cultures, but never in pollen washes. Dephosphorylation (by treatment with alkaline phosphatase) or deglycosylation (by treatment with *N*-acetyl-β-D-glucosaminidase) of the protein extracts prior to electrophoresis did not change the intensity or mobility of the bands (data not shown).

Similar results were obtained using a pool of antisera from patients allergic to birch pollen, instead of BIP 1, as first antibody in the immunoblots (data not shown).

### 3.2. Northern blot analysis

Northern blots were performed to study the expression of *Bet v* 1 at the transcriptional level in birch cell suspension cultures under different experimental conditions (Fig. 2). <sup>32</sup>P-labelled probes, either DNA or anti-sense RNA, corresponding to an internal fragment of the coding region, were prepared from the pollen *Bet v* 1 cDNA clone and used for hybridization (see Materials and methods). The positive control, birch pollen RNA, gave always a strong hybridization signal (lane 1 in all panels). *Bet v* 1 transcripts were not detected in birch leaves (Fig. 2A, lanes 3 and 4; Fig. 2B, lane 2) and only a low, or no signal was observed in control suspension cultures (Fig. 2A, lane 2; Fig. 2B, lane 3; Fig. 2C, lane 2).

As shown in Fig. 2A, clear induction of a transcript hybridizing with the *Bet v* 1 probe was detected when the RNA was prepared from birch cells co-cultivated

with *Pseudomonas syringae* (lane 5). This positive signal was not due to cross-hybridization of the probe with bacterial RNA (data not shown). In addition, the cultures were subjected to different treatments, known to induce specific PR proteins in other systems, such as heat shock (3 h at 42°C, lane 6), metal ions (AgNO<sub>3</sub>, lane 7; LiNO<sub>3</sub>, lane 8; CdCl<sub>2</sub>, lane 10) or chemical elicitors (arachidonic acid, lane 9; salicylic acid, data

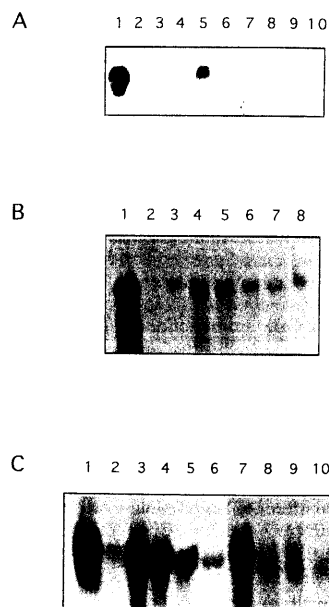


Fig. 2. Northern blot analysis of *Bet v* 1 expression in birch cell suspension cultures under different experimental conditions. (A) Samples contained 10 µg of birch pollen total RNA (lane 1), 25 µg of birch leaf RNA (lanes 3 and 4, two different RNA preparations) or 25 µg of total RNA prepared from cell suspension cultures: non-treated (lane 2), co-cultivated with *P. syringae* for 8 h (lane 3), subjected to a heat-shock treatment (3 h at 42°C, lane 6) or supplemented with 100 µM AgNO<sub>3</sub> (lane 7), 100 µM LiNO<sub>3</sub> (lane 8), 100 µM CdCl<sub>2</sub> (lane 10) or with 1 mg ml<sup>-1</sup> arachidonic acid (lane 9). (B) Samples (15 µg total RNA) were prepared from birch pollen (lane 1), leaves (lane 2) or suspension cultures incubated for 8 h in the absence of microbes (lane 3) or in the presence of *P. syringae* (lane 4), *T. betulina* (lane 5), *X. campestris* (lane 6), *E. coli* (lane 7) or *F. solani* (lane 8). (C) Birch cells were co-cultured for 4, 6, 8 or 25 h with *P. syringae* (lanes 3 to 6, respectively) or with *F. solani* (lanes 7 to 10). Controls: birch pollen (lane 1), control suspension culture (time 0, lane 2). All lanes contained 15 µg total RNA. Hybridization was carried out as described in Methods, using anti-sense RNA (A) or DNA (B and C), <sup>32</sup>P-labelled *Bet v* 1 probes. Each panel corresponds to a single, independent Northern blot experiment. The figure shows the autoradiograms obtained after overnight (A) or 2 days (B and C) exposure at -80°C.

not shown); these stress treatments had no effect on the expression of *Bet v* 1-related mRNAs.

In an independent Northern blot experiment (Fig. 2B), the induction of *Bet v* 1 transcripts in cells cultured for 8 h in the presence of *P. syringae* (lane 4) or, to a lower extent, of other bacteria (*X. campestris*, lane 6; *E. coli*, lane 7) or fungi (*T. betulina*, lane 5; *F. solani*, lane 8), was confirmed.

Kinetic experiments revealed the rapid induction of the *Bet v* 1 transcript by microbial pathogens, and maximum levels were already observed in the samples withdrawn after 4 h of culture, decreasing progressively at later times. These results are shown in Fig. 2C for *P. syringae* (lanes 3 to 6) and *F. solani* (lanes 7 to 10). After 8 h of culture in the presence of the microbes, the levels of the *Bet v* 1 RNA had already decreased appreciably, thus explaining the relatively weak induction observed with some of the samples in Fig. 2B.

Induction of this transcript was detected with all bacteria and fungi tested. Although *P. syringae* gave normally the strongest induction, differences in the relative intensity of the bands, between cultures and pathogens, were observed in these experiments.

### 3.3. RNase A / T1 protection assays

Northern hybridization, even with the high stringent conditions used in our experiments, would not allow to distinguish between highly homologous transcripts of similar size. Therefore, RNase protection experiments were carried out to establish whether the pathogen-inducible RNA detected in birch cell suspension cultures corresponded to the pollen cDNA clone or to other members of the *Bet v* 1 gene family. Three labelled anti-sense RNA probes were synthesized by in vitro transcription (Fig. 3A) and independently hybridized to total RNA prepared from birch pollen, from non-treated suspension cultures or from the same cells co-cultivated with the bacteria *P. syringae* or *X. campestris*, or with the fungus *C. lindemuthianum*. After digestion with RNases A and T1, the protected fragments were analyzed by denaturing polyacrylamide gel electrophoresis and autoradiography.

The expected full-length protected probes of 744 nt (probe 1, Fig. 3B and E), 270 nt (probe 2, Fig. 3C and F) and 173 nt (probe 3, Fig. 3D) were detected in the samples hybridized with pollen RNA (lanes 1 in panels B, C and D; lanes 6 in panels E and F). Additional, shorter radioactive bands were also observed in these lanes. They can not be explained by degradation of the radioactive anti-sense probes during the assays (lanes 9 in panels B, C and D; lanes 1 in panels E and F), nor by local melting of the anti-sense/sense hybrids and partial nicking of the probes, since these bands were not detected after hybridization of probe 1 (Fig. 3E, lane 3), or probe 2 (Fig. 3F, lane 3) with the corre-

sponding, in vitro transcribed, sense RNAs. Instead, these protected fragments are most likely due to the presence in pollen of several *Bet v* 1 mRNA species sharing various regions of high homology with the cDNA clone over the whole sequence.

In contrast, the full-length protected probes were not detected with RNA prepared from any of the different suspension cultures. However, using probe 1 as anti-sense RNA (Fig. 3B), a protected fragment of about 260 nt, as well as other, shorter radioactive bands of different intensities, were reproducibly observed in samples containing RNA from the suspension cultures co-cultivated with bacteria (lanes 4 and 7) or fungi (lane 8). The same fragments could be detected as faint bands also in the control culture (lane 2). Hybridization with *X. campestris* RNA did not lead to protection of fragments of the anti-sense probe (lane 6). Similar results were obtained in experiments carried out with probe 2 as anti-sense RNA (Fig. 3C). In this case, a fragment of about 170 nt within the probe was protected against RNase digestion by pathogen-inducible RNAs present in the suspension cultures (lanes 4, 7 and 8). Probe 3, however, was completely degraded in the same conditions (Fig. 3D), indicating that the pathogen-induced transcript does not contain long stretches of high homology to the cDNA clone within the 3'-region covered by this probe. Induction of *Bet v* 1-related transcripts in the presence of *P. syringae* has been confirmed in independent RNase protection assays with probes 1 and 2 (Fig. 3E and F, respectively). As for the samples containing pollen RNA, the controls included in these experiments ruled out the possibility that the observed protected fragments were produced by nicking of the radioactive anti-sense probes.

These results demonstrated that the gene corresponding to the *Bet v* 1 cDNA clone, as well as other *Bet v* 1 genes constitutively expressed in pollen, are not expressed in birch cell suspension cultures under any of the experimental conditions tested. They also strongly suggest that at least one, and probably several mRNAs highly homologous to pollen *Bet v* 1 RNAs (i.e., products of transcription of other members of the same gene family) are present at very low levels in control cultures and transcriptionally activated in the presence of bacteria or fungi.

Taken together, the RNase protection experiments allowed to map the region of homology between the newly detected RNA and the cDNA clone to approx. 260 nt, including the 5'-half of the *Bet v* 1 coding sequence and about 25 bases of the 5' non-translated region (Fig. 3A). Since the RNases can cut short non-homologous sequences and even single mismatches in double-stranded hybrids under the conditions used in our experiments [27], the pathogen-inducible and the pollen specific genes must share an almost identical sequence in this region.

### 3.4. *Bet v 1* does not inhibit microbial growth in vitro

A simple hypothesis to explain the presence in mature pollen of high levels of pathogenesis-related proteins would be to assume that *Bet v 1* has a direct anti-microbial activity. After landing on the stigma, *Bet v 1* proteins would be released from the pollen upon

hydration and thus protect the female reproductive tissues from infection by pathogens during the early phase of pollen tube growth through the stigma.

However, as shown in Fig. 4, addition of birch pollen washes, which contain high amounts of *Bet v 1*, to a liquid culture of *P. syringae* did not inhibit bacterial growth. The same negative results were obtained

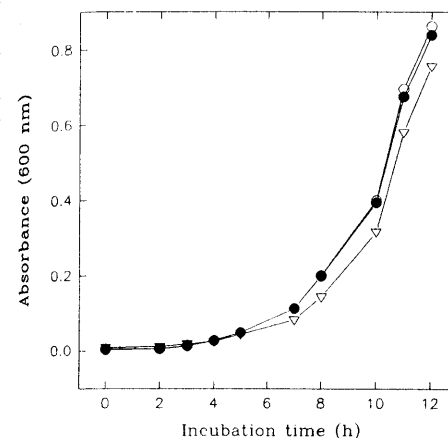


Fig. 4. Effect of birch pollen washes on bacterial growth. A diluted culture of *P. syringae* was divided in three 20 ml aliquots, that were incubated without additions (○) or supplemented with a concentrated birch pollen wash (930 µg protein) (▽) or with the corresponding volume of buffer D (●). At the times indicated, samples (1 ml) were withdrawn to determine bacterial growth by measuring  $A_{600}$ .

with *X. campestris* and *E. coli* and, in one experiment with *P. syringae*, using purified recombinant *Bet v 1* instead of a pollen wash (data not shown). Growth of fungi (*F. solani*, *T. betulina*) on agar plates was also not affected by the presence of birch pollen or concentrated pollen washes (data not shown).

### 4. Discussion

*Bet v 1* homologous proteins from other species are all encoded by more or less complex multigene families [9,11,13,14,16]. Similarly, Southern blot analysis [17]

and I. Swoboda, unpublished results) suggested the presence in the birch genome of at least six *Bet v 1*-related genes. We have recently isolated several cDNA clones corresponding to different pollen *Bet v 1* isoforms (EMBL Nucleotide Sequence Database accession numbers X77200 and X77265 to X77274; Swoboda et al., unpublished data). They all show high homology at the DNA level (more than 90%) over the whole sequence, thus confirming the results of our RNase protection assays. Apart from the genes constitutively expressed in pollen, no other *Bet v 1* clones have been isolated until now. However, the data presented in this paper strongly indicate that a subset of *Bet v 1* genes, sharing very high homology with the pollen-specific members within the 5'-half of the coding region, are transcriptionally activated in the presence of microbial pathogens in cell suspension cultures and, probably, also in birch somatic tissues.

This homology can also be observed at the protein level. Proteins activated by pathogens in cultured birch cells contain the same epitopes recognized by a monoclonal antibody prepared against recombinant pollen *Bet v 1* and by IgEs present in the serum of patients allergic to birch pollen. We do not know the origin of the 18 kDa cross-reacting band observed in the immunoblots. It does not seem to be a phosphorylated or glycosylated form of *Bet v 1*. An antibody against STH-2, the potato homologue of *Bet v 1*, also recognizes a second band of 18 kDa in potato extracts, and it has been demonstrated that it is not a precursor or a modified form of the 17 kDa STH-2 protein [28,29].

The biochemical function of the *Bet v 1* proteins, or their homologues from other plants, whether enzymatic or structural, is not known. *Bet v 1* proteins do not seem to have a direct anti-microbial activity, since pollen washes, containing high levels of *Bet v 1*, did not inhibit bacterial or fungal growth in vitro. Transgenic potato plants, constitutively expressing the endogenous STH-2 gene under control of the CaMV 35S promoter, do not show altered susceptibility to a compatible race

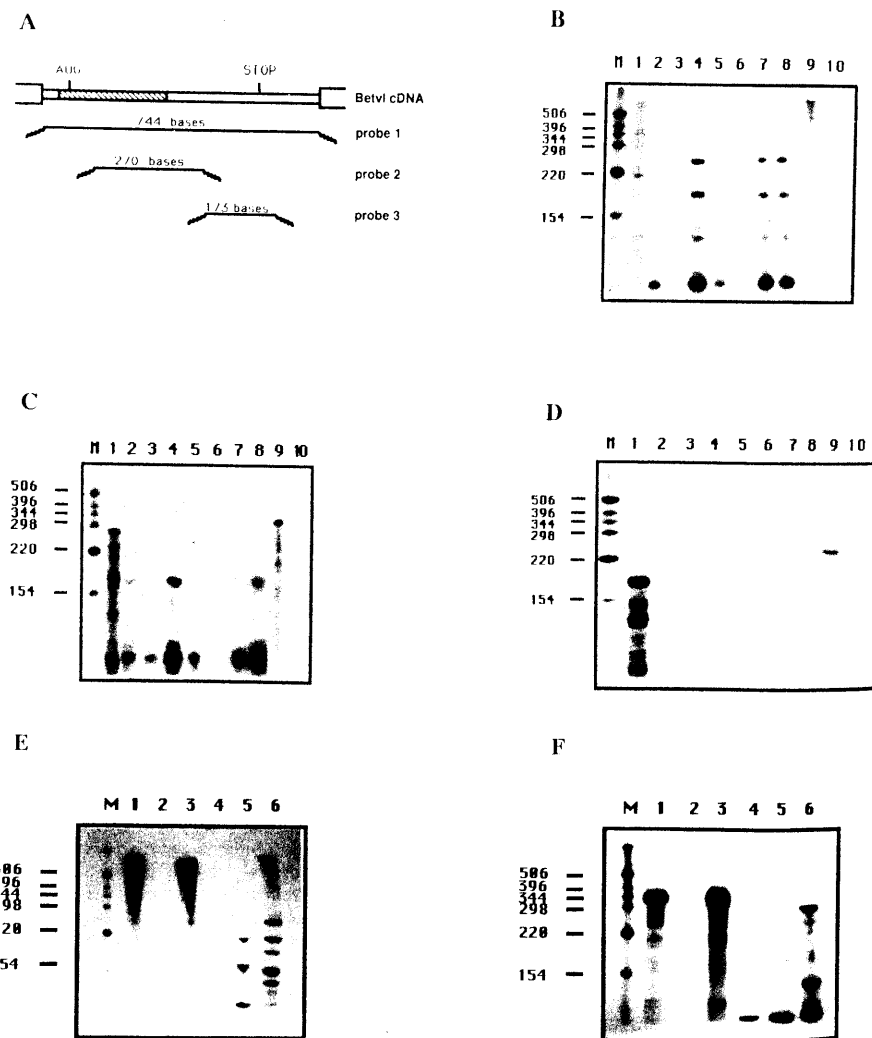


Fig. 3. RNase A/T1 protection experiments. (A) Maps of the *Bet v 1* cDNA clone and anti-sense RNA probes prepared by in vitro transcription of plasmids pG7Betv1A (probe 1), pG2Betv1 (probe 2) and pG7Betv1B (probe 3). For each probe, the length (in number of bases) of the sequence complementary to the cDNA is indicated. Wavy lines at the ends of the probes represent polylinker sequences, contained in the anti-sense transcripts but not hybridizing to *Bet v 1* RNA. The hatched part in the *Bet v 1* cDNA represents the region of homology with the pathogen-inducible RNA detected in suspension cultures. (B, C and D) RNase protection experiments were carried out as described in Materials and methods, using probes 1 (B), 2 (C) or 3 (D) as anti-sense RNA. Samples (in all panels) contained 10 µg total RNA prepared from birch pollen (lane 1), from control cell suspension cultures (lane 2), from cultures treated for 24 h with arachidonic acid (1 mg ml<sup>-1</sup>, lane 3), from cultures co-cultivated with *X. campestris* for 8 h (lane 4) or 24 h (lane 5), with *P. syringae* for 24 h (lane 7) or with *C. lindemuthianum* for 8 h (lane 8), or 10 µg of *X. campestris* total RNA (lane 6). Controls: full-length anti-sense probes, not digested with RNases (lane 9); samples containing the anti-sense probes but not RNA (lane 10). M: size markers, prepared by digestion of pBR322 with *Hinf*I and labelled by filling-in with Klenow DNA polymerase in the presence of [ $\alpha$ -<sup>32</sup>P]dATP; numbers on the left-hand side are the lengths (in nucleotides) of the restriction fragments. The figures show the autoradiograms of the PA/urea gels after exposure for 4 h (lanes M, 1, 9 and 10) or overnight (lanes 2 to 8) at -80°C. (E and F) Probes 1 (E) or 2 (F) were hybridized with 10 µg of total RNA prepared from control suspension cultures (lane 4), from cultures grown for 8 h in the presence of *P. syringae* (lane 5) or from birch pollen (lane 6). Controls: full-length anti-sense probes, not digested with RNases (lane 1); samples containing the anti-sense probes but not RNA (lane 2); anti-sense probes hybridized with 2 µg of the corresponding, in vitro transcribed, sense RNAs (lane 3). Size markers (M) as in panels B, C and D. The gels were exposed overnight at -80°C.

of *Phytophthora infestans* and to potato virus X [29]. In similar experiments, we have produced transgenic tobacco plants containing the pollen *Bet v* I coding sequence cloned behind the 35S promoter and also found no differences in the response against compatible and incompatible bacterial pathogens (*P. syringae*, ssp. *tabaci* and *pisi*, respectively), as compared to control wild-type plants (I. Swoboda, E. Stöger, E. Heberle-Bors, O. Vicente, unpublished results).

From the bacteria and fungi used in our experiments, only *Taphrina betulina* is a natural pathogen of birch. However, all microbes tested, including a non-pathogenic bacterium such as *E. coli*, induced the rapid activation of *Bet v* I genes in cultured birch cells. Since the bands detected in the Northern blots may correspond to several related transcripts, the induction by specific fungi or bacteria of different genes cannot be excluded. In addition, obviously, the results obtained in the artificial situation of the suspension culture cannot be directly extrapolated to the natural interactions of compatible and incompatible pathogens with the whole plant. Nevertheless, these data suggest that *Bet v* I genes belong to the group of genes that are induced unspecifically upon interaction of plant cells with microbes, irrespective of the final result of such interaction (hypersensitive response, systemic infection, etc.), and probably are not directly involved in the defense response against pathogens. Screening of a cDNA library prepared from a *Pseudomonas*-infected cell suspension culture is already in progress in one of our laboratories, in order to isolate putative pathogen-inducible *Bet v* I clones which will be used to investigate the expression of individual genes upon interaction of cultured cells and plant tissues with different compatible and incompatible microbial pathogens.

#### Acknowledgements

This work was supported by the Austrian 'Fonds zur Förderung der wissenschaftlichen Forschung', projects S6002 and S6003.

#### References

- [1] Carr, J.P. and Klessig, D.F. (1989) in Genetic engineering, principles and methods (Setlow, J.K., ed.), Vol. 11, pp. 65-100, Plenum Press, New York.
- [2] Lamb, C.J., Lawton, M.A., Dron, M. and Dixon, R.A. (1989) Cell 56, 215-224.
- [3] Van Loon, L.C. (1985) Plant Mol. Biol. 4, 111-116.
- [4] Bowles, D.J. (1990) Annu. Rev. Biochem. 59, 873-907.
- [5] Dong, X., Mindrinos, M., Davis K.R. and Ausubel, F.M. (1991) Plant Cell 3, 61-72.
- [6] Kauffmann, S., Legrand, M., Geoffroy, P. and Fritig, B. (1987) EMBO J. 6, 3209-3212.
- [7] Kombrink, E., Schröder, M. and Hahlbrock, K. (1988) Proc. Natl. Acad. Sci. USA 85, 782-786.
- [8] Legrand, M., Kauffmann, S., Geoffroy, P. and Fritig, B. (1987) Proc. Natl. Acad. Sci. USA 84, 6750-6754.
- [9] Walter, M.H., Liu, J.-W., Grand, C., Lamb, C.J. and Hess, D. (1990) Mol. Gen. Genet. 222, 353-360.
- [10] Somssich, I.E., Schmelzer, E., Bollmann, J. and Hahlbrock, K. (1986) Proc. Natl. Acad. Sci. USA 83, 2427-2430.
- [11] Somssich, I.E., Schmelzer, E., Kowalleck, P. and Hahlbrock, K. (1988) Mol. Gen. Genet. 213, 93-98.
- [12] Daniels, C.H., Fristensky, B., Wagoner, W. and Hadwiger, L.A. (1987) Plant Mol. Biol. 8, 309-316.
- [13] Fristensky, B., Horowitz, D. and Hadwiger, L.A. (1988) Plant Mol. Biol. 11, 713-715.
- [14] Matton, D.P. and Brisson, N. (1989) Mol. Plant-Microbe Int. 2, 325-331.
- [15] Warner, S.A.J., Scott, R. and Draper, J. (1992) Plant Mol. Biol. 19, 555-561.
- [16] Crowell, D.N., John, M.E., Russell, D. and Amasino, R.M. (1992) Plant Mol. Biol. 18, 459-466.
- [17] Breiteneder, H., Pettenburger, K., Bito, A., Valenta, R., Kraft, D., Rumpold, H., Scheiner, O. and Breitenbach, M. (1989) EMBO J. 8, 1935-1938.
- [18] Murashige, T. and Skoog, F. (1962) Physiol. Plant. 15, 473-497.
- [19] Laemmli, U.K. (1970) Nature 227, 680-685.
- [20] Jarolim, E., Tejkl, M., Rohac, M., Schierka, G., Scheiner, O., Kraft, D., Breitenbach, M. and Rumpold, H. (1989) Int. Arch. Allergy Appl. Immunol. 90, 54-60.
- [21] Bradford, M.M. (1976) Anal. Biochem. 72, 248-254.
- [22] Maniatis, T., Fritsch, E.F. and Sambrook, J. (1982) Molecular cloning: a laboratory manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor.
- [23] Melton, D.A., Krieg, P.A., Rebagliatti, M.R., Maniatis, T., Zinn, K. and Green, M.R. (1984) Nucleic Acids Res. 12, 7035-7056.
- [24] Goodall, G.J., Wiebauer, K. and Filipowicz, W. (1990) Methods Enzymol. 181, 148-161.
- [25] Chomczynski, P. and Sacchi, N. (1987) Anal. Biochem. 162, 156-159.
- [26] Fourney, R.M., Miyakoshi, J., Day III, R.S. and Paterson, M. (1988) Focus 10, 5-7.
- [27] Myers, R.M., Larin, Z. and Maniatis, T. (1985) Science 227, 1242-1246.
- [28] Constabel, C.P. and Brisson, N. (1992) Planta 188, 289-295.
- [29] Constabel, C.P., Bertrand, C. and Brisson, N. (1993) Plant Mol. Biol. 22, 775-782.



F

I

\* Divis

\* Molecu

#### Abstract

In or  
renin, tr  
promote  
chloram  
promote:  
the -25  
noted fo  
chorio-de  
in chorio  
in the co  
activity in  
this cell t  
for huma

#### Keywords

#### I. Introd

Renin  
pease  
zyme  
stensen  
direct p  
phic a  
eulatin  
ithelic  
.01%  
huma  
ed m

resp  
Lau.02

81/9  
167.