

Expression of Bet v 1, the major birch pollen allergen, during anther development

An in situ hybridization study

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Summary. Bet v 1 proteins, a group of isoforms that constitute the major pollen allergen of the white birch (*Betula verrucosa*), are encoded by a complex multigene family which also includes a subset of genes transcriptionally activated in cell suspension cultures in the presence of microbial pathogens. We have investigated the temporal and spatial expression patterns of *Bet v 1* genes during development of male birch inflorescences. Western and Northern analysis indicated that the expression of Bet v 1 isoallergens is regulated at the transcriptional level and activated only during the late phases of anther maturation, so that high levels of these proteins accumulate in mature pollen. In situ hybridization experiments, carried out with sections of male inflorescences isolated at defined developmental stages, showed that *Bet v 1* genes are specifically expressed in late bicellular and mature pollen, but no transcripts were detected in sporophytic anther tissues of any developmental stage. These data suggest a possible role of Bet v 1 proteins during pollen germination on the stigma, protecting the female reproductive tissues from infection by pathogens.

Keywords: *Betula verrucosa*; Bet v 1 proteins; In situ hybridization; Pathogenesis-related proteins; Pollen allergens; Pollen development.

Abbreviations: BCIP 5-bromo-4-chlor-3-indolylphosphate; BSA bovine serum albumin; DIECA sodium diethyldithiocarbamate; NBT 4-nitro blue tetrazolium chloride; PMSF phenylmethylsulfonyl fluoride.

Introduction

Pollen of the white birch (*Betula verrucosa*) is one of the main causes of Type I allergic reactions in large

parts of the northern hemisphere. Bet v 1, a polypeptide of 17 kDa, has been identified as the major birch pollen allergen, responsible for IgE binding in more than 95% of patients allergic to birch pollen (Ipsen and Loewenstein 1983). As for other allergenic proteins, the high solubility of Bet v 1 in aqueous systems allows it to easily cross the mucous barriers of the respiratory track, evoking the IgE-mediated release of inflammatory mediators and thus causing the well-known symptoms of allergic rhinitis, rhinoconjunctivitis and asthma bronchiale. Bet v 1 proteins are encoded by a complex multigene family. A *Bet v 1* cDNA clone was initially isolated from a pollen expression library by immunoscreening with a patient's serum (Breiteneder et al. 1989), and we have recently cloned several cDNAs by PCR, encoding additional pollen Bet v 1 isoforms (EMBL Nucleotide Sequence Database accession numbers X77200 and X77265 to X77274; Swoboda et al. 1995). Their deduced amino acid sequences show high homology to a new class of conserved pathogenesis-related (PR) proteins, that are induced by microbial pathogens in bean (Walter et al. 1990), parsley (Somssich et al. 1988), pea (Fristensky et al. 1988), or potato (Matton and Brisson 1989). In agreement with this sequence homology, we have also shown that a subset of *Bet v 1* genes, different from the members of the gene family constitutively expressed in pollen, are transcriptionally induced in birch cell suspension cultures in the presence of bacterial or fungal pathogens (Swo-

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boda et al. 1994). These data suggested a possible functional role of Bet v 1 proteins in pathogen resistance of pollen.

As for other allergens, studies on Bet v 1 have focused mainly on its immunological properties and possible diagnostic or therapeutical applications (Ferreira et al. 1993, and references therein), and very little is known about the expression patterns and biological function of these proteins during anther development. In birch, male flowers, each containing around 20,000 pollen grains, are morphologically very simple, as they only consist of two to three tepals and two deeply divided stamens. The flowers are set in loose, hanging catkins, that start to develop in late spring. Usually, microsporocytes undergo meiosis at the beginning of autumn, pollen development starts in late autumn and, after a period of dormancy in winter, continues in spring.

In this communication we describe the expression of pollen *Bet v 1* genes at different stages of anther development, defined on the basis of length measurements and morphological markers of the male inflorescences. Western and Northern analysis indicate that the expression of Bet v 1 proteins is activated, at the transcriptional level, only during the final phase of pollen maturation. However, correlation of changes in external morphology with the presence of microspores or pollen grains at specific developmental stages is difficult, as pollen development does not take place synchronously within a pollen sac, and anthers at different stages of development can be found in a single inflorescence. Therefore, these results have been confirmed by *in situ* hybridization experiments, which also showed that Bet v 1 isoforms are specifically expressed in mature pollen, but not in sporophytic tissues of the anther.

Material and methods

Plant material

Whole male inflorescences of different developmental stages were collected from three selected birch trees in Vienna, immediately frozen in liquid nitrogen and stored at -80°C until used. Inflorescences were classified into five developmental stages on the basis of length, morphological criteria and developmental events occurring within the anthers (Table 1; see also Figs. 3 and 4). Green inflorescences of the first stage had a length of 1 to 1.5 cm and microsporocytes within the anthers were just before or at the beginning of meiosis. The second stage was defined as the period when tetrads and very early microspores were present in the anthers; the size of the inflorescences was between 2.5 cm and 3.2 cm. Anthers of the third stage contained uninucleate microspores and early to mid-bicellular pollen and the inflorescences, of 3.2 to 4 cm in length, were still green. In the fourth, or "premature" stage, slightly yellow anthers contained

late bicellular pollen grains of various sizes, many of them already in the process of dehydration; inflorescences had a length of 4 to 5 cm. Finally, inflorescences of the fifth stage ("mature") had enlarged to a size of 6 to 7 cm and contained anthers of a strong yellow colour, just before anthesis, filled with fully mature pollen of homogeneous size. Samples of stages one to three were collected in autumn, and premature and mature inflorescences during the following spring.

Protein extracts and immunoblotting

Isolated mature birch pollen, obtained from Allergon AB (Engelholm, Sweden), was suspended in 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 . The sample was centrifuged at 10,000 rpm for 5 min, at 4°C , in a Sorvall ss34 rotor. The supernatant, referred to as "pollen wash", was concentrated by ammonium sulfate precipitation (80% saturation) and dialyzed against buffer D (17.5 mM potassium phosphate buffer, pH 7.0). Proteins not released from the pollen grains by this treatment were recovered as follows: After washing in buffer E, the pollen pellet was ground in a mortar to a fine powder and extracted in buffer E supplemented with 100 mM NaCl and a mixture of protease inhibitors (0.1 mM PMSF, 1 mM benzamidine, 2 $\mu\text{g}/\text{ml}$ aprotinin, 2 $\mu\text{g}/\text{ml}$ leupeptin, 100 $\mu\text{g}/\text{ml}$ bacitracin, 1 $\mu\text{g}/\text{ml}$ antipain). The homogenate was centrifuged at 15,000 rpm for 20 min, at 4°C , in a ss34 rotor, and the supernatant, after ammonium sulfate precipitation, was extensively dialyzed against buffer D; this sample was referred to as "washed-pollen extract". Extracts from whole male inflorescences, collected at different developmental stages, and from non-treated isolated mature pollen ("whole pollen extract") were prepared as described above for the "washed-pollen extract". All extracts were stored in aliquots at -80°C until used. Protein concentration in the different samples was measured by the method of Bradford (1976) using the Bio-Rad reagent and BSA as standard.

For immunoblotting, polypeptides were separated in 15% PA/SDS gels (Laemmli 1970) and transferred to nitrocellulose membranes by semi-dry blotting (Semi-Phor TE70 transfer unit, Hoeffer). Bet v 1 proteins were then detected incubating the membranes with the monoclonal antibody PB1, prepared against pollen Bet v 1 (Jarolim et al. 1989), followed by an anti-mouse antibody coupled to alkaline phosphatase and the colorimetric substrates BCIP and NBT.

Synthesis of nucleic acid probes

A 270-bp-long Bam HI/Hin dIII fragment from the coding region of the pollen *Bet v 1* cDNA clone (Breiteneder et al. 1989), which had been labelled with ^{32}P by random oligonucleotide priming (Maniatis et al. 1982), was used as hybridization probe in Northern blots. The same fragment, as well as a 173 bp Bam HI/Afl III fragment, containing coding and non-coding sequences from the 3'-end of the *Bet v 1* cDNA clone, were subcloned into pGEM vectors (Promega), giving rise to plasmids pG2 and pG7, respectively. Sense and antisense, ^{35}S -labelled RNA probes were synthesized from these constructs by *in vitro* transcription (Melton et al. 1984), and used in *in situ* hybridization experiments; probe pG2 was previously subjected to partial hydrolysis in alkali, by incubation for 30 min, at 60°C , in the presence of one volume of 80 mM NaHCO_3 and 120 mM Na_2CO_3 , pH 10.2.

RNA preparation and Northern blot analysis

Total RNA was isolated from whole male inflorescences according to the method of Yeh et al. (1991). For Northern blots, RNA was

separated in 1.2% agarose/formaldehyde gels and transferred to nylon membranes (Hybond-N, Amersham) by capillary blotting. Prehybridization (1–4 h) and hybridization (20–24 h) were performed at 42 °C in 25 mM potassium phosphate buffer, pH 7.4, 5× SSC, 5× Denhardt's solution, 50% formamide and 100 µg/ml sonicated salmon sperm DNA. The membranes were washed twice in 2× SSC, 0.1% SDS at room temperature for 20 min, and once in 0.1× SSC, 0.1% SDS at 55 °C for 30 min. Radioactive bands were detected by autoradiography in Kodak AR X-Omat films.

In situ hybridization

Whole birch inflorescences were fixed overnight in ice-cold 100 mM sodium phosphate buffer, pH 7.0, containing 2% paraformaldehyde and 0.2% glutaraldehyde. The samples were dehydrated in graded ethanol and tertiary butanol series and embedded in paraffin. Sections, 8 µm thick, were attached to poly-L-lysine-coated slides, deparaffinized with xylene and rehydrated through graded ethanol series. Slides with anther sections were subsequently pretreated with 1 µg/ml proteinase K in 100 mM Tris-HCl, 50 mM EDTA, pH 7.5, at 37 °C for 30 min, followed by 0.25% acetic anhydride in 100 mM triethanolamine, pH 8.0, at room temperature for 15 min, then dehydrated in graded ethanol series and air-dried. Vacuum dried sections were hybridized with ³⁵S-labelled, sense or anti-sense, pG2 or pG7 RNA probes for 2 to 18 h, at 50 °C, in a solution containing 50% deionized formamide, 5× SSPE (20× SSPE is 3 M NaCl, 200 mM NaH₂PO₄, 20 mM EDTA, pH 7.4), 10% dextran sulfate, 1% polyvinylpyrrolidone, 5× Denhardt's solution, 0.2% SDS, 100 mM DTT and 100 µg/ml denatured herring sperm DNA. After hybridization, the slides were rinsed in 2× SSPE, containing 0.1% SDS, washed first in 2× SSPE, 50% formamide, 10 mM DTT, at 50 °C, and then in 2× SSPE, 10 mM DTT, at room temperature. The samples were treated for 1 h at 37 °C in the presence of 20 µg/ml ribonuclease A, followed by incubation at 50 °C in 0.1× SSPE, 50% formamide, 10 mM DTT, and then immersed in 0.1× SSPE, 10 mM DTT, at room temperature. Finally, the slides were dehydrated in graded ethanol series (supplemented with 0.3 M ammonium acetate), followed by 100% ethanol, coated with Amersham nuclear emulsion and exposed for 3 to 7 days at 4 °C. After development in Kodak D 19

developer and fixation in Kodak fixer, sections were stained with 0.05% toluidine blue O and mounted with DPX. Pictures were taken with a Zeiss Axioskop.

Results

Expression of Bet v 1 proteins in male birch inflorescences of different developmental stages

To determine the temporal expression of Bet v 1 proteins during anther development, extracts from whole inflorescences of different developmental stages (see Material and methods and Table 1) were analyzed by Western blots (Fig. 1), using a monoclonal antibody, BIP 1, prepared against Bet v 1 (Jarolim et al. 1989). No protein could be detected by the antibody in

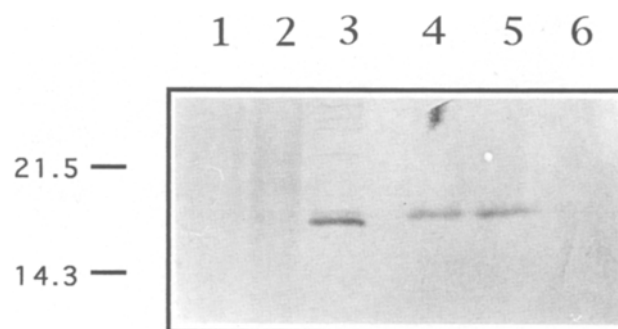


Fig. 1. Expression of Bet v 1 proteins during birch anther development. Immunoblot of protein extracts prepared from whole male inflorescences of the second (1), third (2), and fifth (3) developmental stage or from isolated mature pollen ("whole pollen extract"; 4); 5 "pollen wash", 6 corresponding "washed-pollen extract". Samples from whole inflorescences all contained 10 µg of protein. Protein loaded in 4–6 was obtained by extracting or washing equal amounts of mature pollen (54 µg). The blot was developed with the anti-Bet v 1 monoclonal antibody BIP 1. Numbers on the left-hand side indicate the position and molecular mass (in kDa) of protein standards

Table 1. Major events during birch anther development

Developmental stage of male inflorescence	Inflorescence length (cm)	Pollen development within the anther
Stage 1	1.0–1.5	microsporocytes before or at the beginning of meiosis
Stage 2	2.5–3.2	meiosis in progress, tetrads and very early microspores
Stage 3	3.2–4.0	uninucleate and mitotic microspores, early to mid-bicellular pollen
Stage 4 ("premature")	4.0–5.0	late bicellular pollen grains starting dehydration
Stage 5 ("mature")	6.0–7.0	anthers just before anthesis, filled with mature pollen

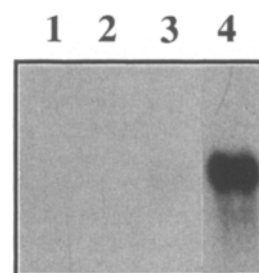


Fig. 2. Northern blot analysis of *Bet v 1* gene expression during anther development. Samples contained 20 µg total RNA isolated from whole male inflorescences at stage 1 (1), stage 2 (2), stage 3 (3) or from mature inflorescences (stage 5; 4). The figure shows the autoradiogram of the Northern blot after overnight exposure at –80 °C

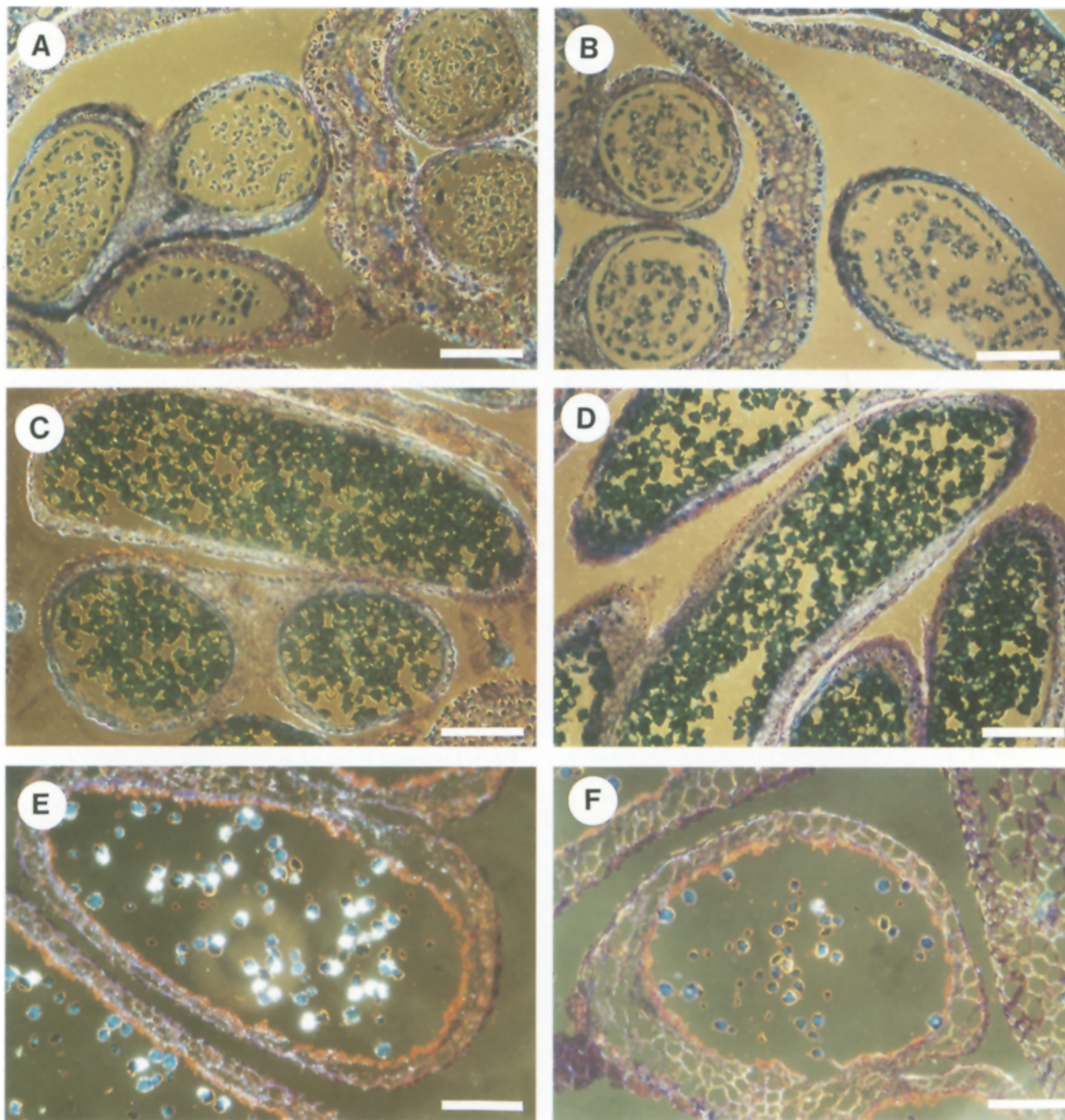


Fig. 3 A–F. Localization of Bet v 1 mRNAs during birch anther development. In situ hybridization of ^{35}S -labelled, anti-sense (A, C, and E) or sense (B, D, and F) pG2 RNA probes with anthers of the second (A and B), third (C and D), and fourth (E and F) developmental stage. All panels show dark-field micrographs of the samples. The presence of labelled RNA/RNA hybrids in late bicellular pollen (E) is observed as white silver grains under dark-field microscopy. Bars: 100 μm

young stages of anther development (Fig. 1, lanes 1 and 2), but high levels of Bet v 1 were found in mature inflorescences (lane 3). Whole pollen extracts, prepared from isolated mature pollen, also contained the 17 kDa band reacting with the antibody (Fig. 1,

lane 4), but Bet v 1 was quantitatively released from the pollen upon incubation in an aqueous buffer (lane 5) and could not be detected in the “washed-pollen extract” (lane 6). Protein samples loaded in lanes 4–6 (Fig. 1) were prepared by extracting or washing the

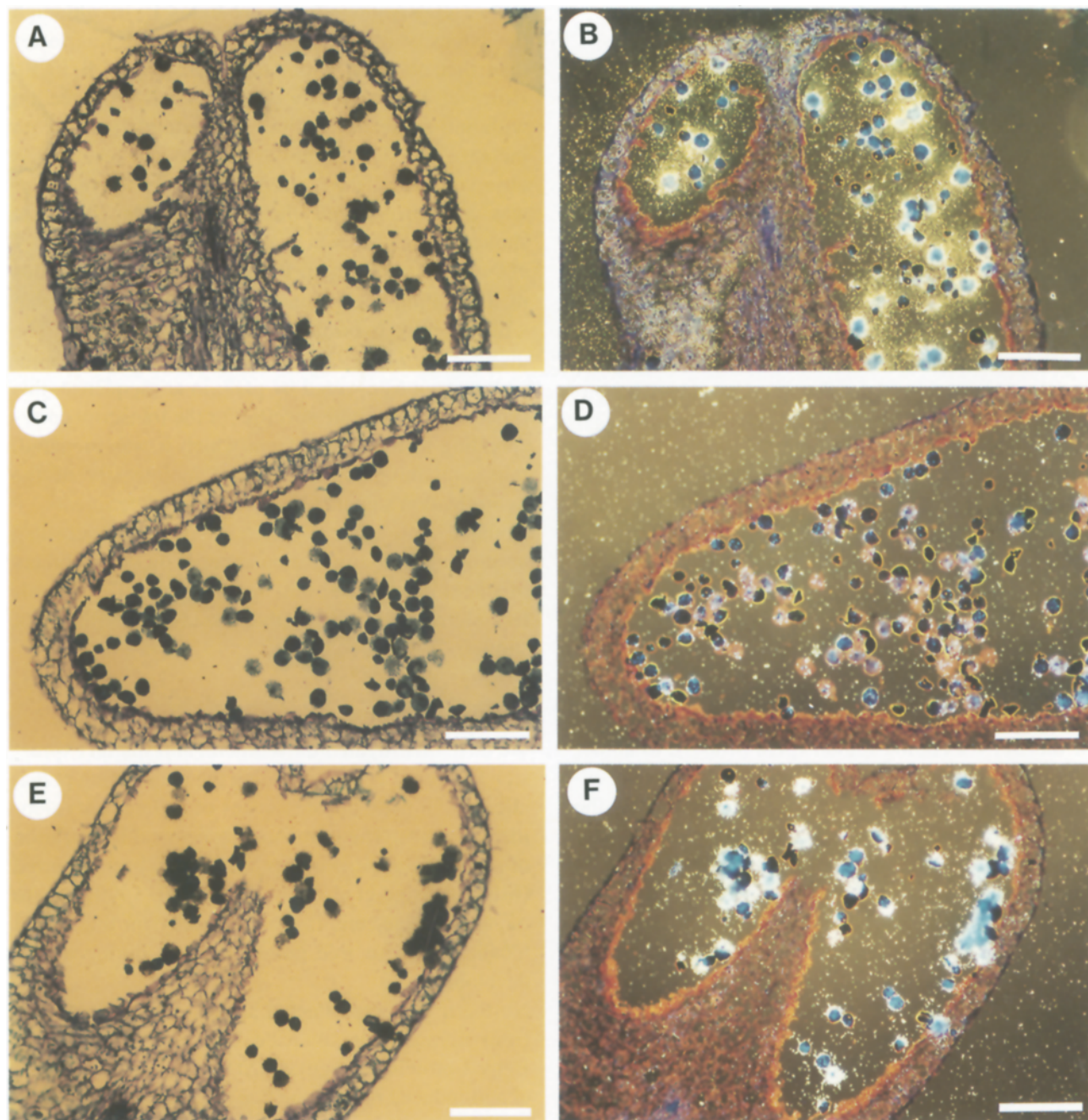


Fig. 4 A–F. Localization of Bet v 1 mRNAs in mature birch anthers. In situ hybridization experiments were carried out with Bet v 1 anti-sense (A and B) or sense (C and D) pG2 probes, or with the anti-sense pG7 probe (E and F). A, C, and E Bright-field microscopy; B, D, and F corresponding dark-field micrographs of the same samples. White silver grains can be observed in B and F, revealing the presence of labelled RNA/RNA hybrids in mature pollen. Bars: 100 μ m

same amounts of pollen, so that the levels of Bet v 1 could be directly compared.

These results indicate that Bet v 1 isoforms are expressed only in mature inflorescences but not at earlier stages of development, and that they are

present in mature pollen grains. Obviously, these experiments do not exclude the possibility that these proteins are also expressed in sporophytic tissues of mature anthers.

Bet v 1 gene expression is regulated at the transcriptional level

To determine whether the expression of the allergen was regulated at the transcriptional or post-transcriptional level, Northern blots were performed with RNA isolated from male inflorescences at different developmental stages, using a ^{32}P -labelled DNA probe corresponding to an internal fragment of the Bet v 1 coding region (Fig. 2). High amounts of Bet v 1 transcripts were detected in mature anthers (Fig. 2, lane 4), but not at earlier developmental stages (lanes 1–3). These results corresponded to those obtained in Western blots and we concluded therefore that *Bet v 1* gene expression is transcriptionally regulated during the development of male inflorescences.

In situ hybridization

In situ hybridizations with two different single-stranded Bet v 1 ^{35}S -RNA probes, were carried out in sections of male inflorescences to confirm the temporal gene expression pattern observed in Northern blots and to localize the transcripts in specific anther tissues and cell types.

Figure 3 shows in situ hybridization of anti-sense or sense probes, corresponding to an internal fragment of the Bet v 1 coding region (pG2 probes), to anther sections of stages two to four. No signals above background levels were observed after hybridization of the anti-sense probe with very young inflorescences of stage one (data not shown) or with anther tissue of the second (Fig. 3 A) or third stage (Fig. 3 C). However, as predicted from Northern blot experiments, the corresponding mRNAs accumulated at later stages of birch anther development. A relatively strong hybridization signal, only localized in pollen grains, was observed in premature anthers (stage four), containing late bicellular pollen (Fig. 3 E). Specific hybridization signals were not observed in any of the samples in the controls with the sense pG2 RNA probe (Fig. 3 B, D, and F).

Sections of fully mature inflorescences of the fifth stage were also hybridized with the same pG2 anti-sense (Fig. 4 A, B) or sense (Fig. 4 C, D) probes. The Bet v 1 mRNA hybridization signals intensified, as compared to the premature stage, but remained localized in the pollen grains, and no silver grains above background levels were observed in any other anther tissue. Identical, pollen-specific localization patterns were observed with the pG7 anti-sense RNA probe, containing 3' coding and non-coding sequences of the

Bet v 1 cDNA clone (compare Fig. 4 E, F), although the intensity of the hybridization signal was lower than that obtained with probe pG2 (Fig. 4 B to F). However, the specificity of Bet v 1 mRNA detection with anti-sense pG7 probe was demonstrated, as no increase in signal above background levels was observed when the corresponding sense transcript was hybridized with the anther tissue (data not shown). The different sequences of pollen *Bet v 1* cDNAs (Swoboda et al. 1995) are highly homologous in the coding region, including the pG2 probe that will therefore hybridize to all Bet v 1 mRNAs. The observed homology is lower in the 3' coding and non-coding regions covered by pG7; in consequence, this probe should be more specific for the transcript corresponding to the cDNA from which it was prepared, thus explaining the differences in intensity of the hybridization signals observed in our experiments.

Both pG7 anti-sense and sense RNA probes were also used in in situ hybridization experiments with anthers of stages one to three but, as for probe pG2, no positive signals were detectable in any case (data not shown).

Discussion

We have investigated the temporal and spatial expression of *Bet v 1* genes, encoding the major birch pollen allergen, in male inflorescences of different developmental stages. The immunocytochemical localization of some allergenic proteins has already been reported (Miki-Hirosige et al. 1994, Staff et al. 1990) but, to our knowledge, this is the first study on the in situ localization of a tree pollen allergen during anther development.

Northern blot experiments as well as data obtained from immunoblots indicate that the expression of Bet v 1 proteins is regulated at the transcriptional level and induced only at the late stages of anther development, shortly before anthesis; Bet v 1 mRNAs and the corresponding isoallergens then accumulate massively in mature pollen. These proteins are readily and quantitatively released from pollen in the presence of aqueous buffers. Since no signal peptide could be identified in the sequences of the isolated cDNA clones (Breiteneder et al. 1989, Swoboda et al. 1995), it should be assumed that Bet v 1, together with other soluble cell constituents (Simon 1974), passively leaks out in a few minutes upon pollen hydration, before the integrity of the plasma membrane is reestablished.

This high solubility excludes the use of conventional immunocytochemical methods for the localization of Bet v 1 proteins, and special fixation techniques in anhydrous solvents had to be employed to show their presence in the cytoplasm of mature birch pollen (Grote 1992). Our in situ hybridization experiments, on the contrary, allowed to establish precisely the temporal and spatial patterns of *Bet v 1* gene expression during anther development, which is predominantly, if not exclusively, restricted to late bicellular and mature pollen. No Bet v 1 transcripts were detected in sporophytic anther cell types of any developmental stage, including the tapetum, from which many of the outer pollen wall proteins originate. The tapetum has been regarded as a general source of proteins responsible for allergy (Knox et al. 1970, Knox and Heslop-Harrison 1971) which apparently is not true for Bet v 1.

The genes encoding Bet v 1 isoallergens can be considered "late" pollen-specific genes (Mascarenhas 1990), that are transcriptionally activated in late bicellular pollen, but not expressed at earlier developmental stages. This expression pattern provides some insights into the possible biological function of Bet v 1 proteins in pollen. Most likely, they are not involved in the formation of the male gametophyte, unless they have a protective role in the dehydration process that takes place during the stages in which *Bet v 1* genes are expressed, just before anthesis. Alternatively, the accumulation of high amounts of Bet v 1 proteins in fully mature, dry pollen, suggests that they might act after pollen shedding, i.e., during pollen germination in the stigma.

On the basis of sequence homology to a class of conserved pathogenesis-related proteins identified in other plant species, it has been proposed that Bet v 1 could be involved in defense reactions of pollen against pathogen attack (Breiteneder et al. 1989). Our recent finding that a subset of *Bet v 1* genes is transcriptionally activated in birch cell suspension cultures in the presence of microbial pathogens (Swoboda et al. 1994) lends support to this idea. After landing on the stigma, Bet v 1 proteins would be released from the pollen upon hydration and, assuming that they have an anti-microbial activity, thus protect the female reproductive tissues (rather than pollen itself) from infection by pathogens during the early phase of pollen tube growth. Alternatively, or additionally, Bet v 1 could be required for the growth of the pollen tube through the stigma, a process that shows analogy, and

has been proposed to have a common evolutionary origin, to the interactions between pathogens and host organisms (Dickinson 1994).

The biochemical function of Bet v 1, or its homologues from other plants, whether enzymatic or structural, is still unknown. Once established, it will hopefully provide essential information about the role of these proteins in the defense reactions against pathogens, as well as for pollen development, male gametophytic function and/or pollen-pistil interactions.

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