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A Model System to Study the Environment-Dependent Expression of the *Bet v 1a* Gene Encoding the Major Birch Pollen Allergen

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Abstract
Background: The major birch pollen allergen Bet v 1 (or Bet v 1a) is one of the main causes of seasonal type I allergies. Various environmental factors such as light, temperature and air pollution may influence the activity of the *Bet v 1a* gene. The creation of a model system to evaluate the role of environmental factors affecting the *Bet v 1a* gene expression would be highly desirable. We suggest the use of transgenic tobacco plants carrying a *Bet v 1a* promoter-reporter gene fusion as such a system. ***Methods:*** The promoter of the *Bet v 1a* gene was isolated with the use of the Universal Genome Walker kit (BD Biosciences Clontech, USA). Web Software was used to search for putative *cis*-regulatory elements within the promoter. Transgenic tobacco plants harboring the promoter-β-glucuronidase *(GUS)* reporter gene fusion were obtained via *Agrobacterium tumefaciens*-mediated transformation. Promoter activity was examined with histochemical and quantitative assays. ***Results:*** Structural analysis predicted elements responsible for pollen-specific, light-, stress- and hormone-mediated induction within the *Bet v 1a* promoter. The evaluation of GUS activity in transgenic tobacco plants showed that the *Bet v 1a* promoter is pollen-specific. Moreover, the *Bet v 1a* promoter is considered to be the strongest isolated pollen-specific promoter reported to date. It was shown that temperature and abscisic acid positively regulate the activity of the *Bet v 1a* promoter during pollen development, providing evidence for environment-dependent regulation of the *Bet v 1a* gene. ***Conclusions:*** A model system to study the effect of environmental factors on the expression of the *Bet v 1a* gene encoding the major birch allergen in pollen was generated. Additionally, we suggest that this system could be used to search for factors that inhibit the activity of the gene in pollen in order to reduce the potential allergenicity of birch trees.

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Introduction

Pollen from trees of the order *Fagales* (birch, oak, alder, hazel, hornbeam) are major causes of type I allergies in early spring. In urban areas, approximately 7% of the population suffer from type I allergies caused by these pollen [1]. The major birch (*Betula verrucosa* Ehrh. syn. *Betula pendula* Roth.) pollen allergen Bet v 1 represents

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the predominant inducer of tree pollen-allergic response [2], since almost 96% of birch pollen-allergic patients show IgE binding to Bet v 1 and 60% of these patients react exclusively to this protein [1]. Moreover, proteins homologous to Bet v 1 were identified in different plant-derived foods, providing molecular evidence for the cross-reactivity of tree pollen allergy and allergy to foods of plant origin [for a review, see 3].

The gene encoding the Bet v 1a protein was originally discovered by screening a pollen cDNA library with IgE serum from patients allergic to birch pollen [4], and meanwhile many of the immunological, biochemical and biological features of the protein have been studied. Modern diagnostics of allergic diseases and new therapeutic approaches are based on the use of recombinant allergens [5]. It was shown that *Escherichia coli* and *Nicotiana benthamiana* expression systems could be used to produce recombinant allergens with the assigned properties [5, 6]. Furthermore, analysis of the 3-dimensional structure identified potential B cell epitopes on the molecular surface of the Bet v 1 protein and thus provided a structural basis for the reactivity of the molecule with IgE antibodies [7]. Based on these data genetically engineered hypoallergenic derivatives with modified B cell epitopes have been produced, which cannot bind to IgE antibodies anymore and might therefore be used as vaccines against birch pollen allergy [8].

The *Bet v 1* gene family displays a considerable degree of heterogeneity [9] and the *Bet v 1* corresponding to the major allergen [4] was referred to as *Bet v 1a* (http://www.allergen.org/Isoall.htm). Swoboda et al. [10] described 13 different Bet v 1 isoforms, termed Bet v 1b to Bet v 1n. N-terminal sequence analysis and mass spectroscopy of purified natural Bet v 1 showed that isoforms a and b represent about 35% of the Bet v 1 pool in pollen [11]. Apart from those proteins expressed in pollen, three isoforms, termed Bet v 1-Sc1, Bet v 1-Sc2 and Bet v 1-Sc3, have been discovered that are induced in somatic cells upon their interaction with microorganisms and fungi [12]. Up to now 37 members have been entered in the list representing the *Bet v 1* gene family (http://www.allergen.org/Isoall.htm).

Bet v 1 isoforms were included in the class of intracellular pathogenesis-related (PR) proteins (IPR or PR-10 proteins) on the basis of amino acid sequence homology, protein size and total charge [for a review, see 13]. Bet v 1 proteins were proposed to be ribonucleases [14, 15], although recent evidence suggests that they may function as a plant steroid carriers [16].

The frequency of allergies depends on many factors including patient susceptibility and various seasonal conditions, which influence both allergen accumulation in pollen and allergen molecule release from pollen grains [17]. Studies on birch trees and forests grown in various ecotopes have shown that environmental factors such as duration of the light period, light intensity, seasonable air and soil temperature, atmosphere precipitation and air pollution may influence the allergen accumulation in birch pollen [18–20]. The increase of birch pollen allergies in industrial countries could be explained as being caused by air pollutants affecting both the health status of the population and allergen accumulation in pollen [21]. Therefore, a study of the impact of environmental factors on pollen allergenicity would be of high importance for epidemiological analyses, patient counseling, and efforts to curb the release and spread of pollen and allergen. Furthermore, immunological and biological research could greatly benefit from an experimental system focused on a particular aspect, in which other factors causing a variation in allergen accumulation and allergic response are eliminated. The aim of the present work was to create a biological model system in order to monitor the contribution of various environmental factors on the induction of the gene encoding the major birch pollen allergen Bet v 1 (syn. Bet v 1a). The necessary prerequisites to construct such a model system were the isolation of the *Bet v 1a* gene promoter and evaluation of its activity in transgenic plants, harboring the promoter fused with a reporter gene. As a reporter, the *E. coli GUS* gene was considered to be suitable rather than fluorescent proteins [22] since the *GUS* fusion system is highly sensitive, easy to quantitate [23] and therefore allows to determine even minor activities of the tested promoter.

Materials and Methods

Plant Material

Nicotiana tabacum cv. Petite Havana SR1 was used as donor plant material. Wild-type and transgenic plants were grown in the greenhouse at 25 °C and 16 h of daylight.

Isolation of the Promoter Region

Genomic DNA was extracted from fresh, young leaves of *B. verrucosa* according to Kim et al. [24]. Fragments supposed to contain the *Bet v 1a* promoter region were amplified from the genomic DNA with the Universal Genome Walker Kit (BD Biosciences Clontech, USA) using adapter primers provided by the kit and the oligonucleotide 5′-CGTAATTGAAAACACCCATGATGGCCTG-3′ as the gene-specific primer derived from the known 5′-terminus of the *Bet v 1a* cDNA [4]. The generated PCR fragments were cloned in the T/A vector pCR2.1 (Invitrogen, USA). Short-run sequencing was

performed with randomly chosen clones to select one, which contained the sequence overlapping the 5′-terminus of the *Bet v 1a* cDNA.

Analysis of the Promoter Sequence

Sequencing was performed using ALF semiautomatic DNA sequencer (Amersham Biosciences, UK). Sequence identity additionally was determined with the use of the NCBI BLAST Service program (http://www.ncbi.nlm.nih.gov/BLAST/) [25]. To examine the integrity of the obtained fragment and that it is not a cloning artifact, the fragment was reamplified from genomic DNA, using gene-specific primer and the primer 5′-CCTAGCCAGTGGGATAAGAGAGGTAGTCTC-3′ generated from the opposite end of the initially obtained sequence, cloned and analyzed. Searches for putative *cis*-acting regulatory elements were carried out with the programs PLACE Web Signal Scan (http://www.dna.affrc.go.jp/htdocs/PLACE/signalscan.html) [26] and PlantCARE (http://oberon.rug.ac.be:8080/PlantCARE/) [27].

Plasmid Construction

The shuttle vector pBI101.1 harboring the β-glucuronidase *(GUS)* gene and the cassette for kanamycin resistance [23] was used to generate the construct in order to evaluate the promoter activities. A *Hin*dIII/*Xho*I fragment containing the *Bet v 1a* promoter region was cut out from the plasmid pCR2.1 and inserted into *Hin*dIII/*Sal*I-cut vector pBI101.1 in order to bring the promoter ATG codon in the frame with the ATG of the reporter gene.

Plant Transformation

The construct was transferred to the *Agrobacterium tumefaciens* strain LBA4404 by electroporation [28]. Tobacco plants were transformed with this strain via leaf disc transformation essentially as described by Horsch et al. [29] and transgenic shoots were selected in Murashige and Skoog (MS) medium [30] containing kanamycin. After root generation, plantlets were transferred to soil for further growth in the greenhouse under the conditions mentioned above. Seeds obtained from the self-pollinated transgenic plants were subjected to segregation analysis to determine the copy number of the inserted chimeric gene. Only homozygous lines with one copy of the transgene per haploid genome (since haploid microspores and mature pollen were examined as well) were taken for further experiments to avoid a possible cosuppression effect of multiple transgene dosage which might alter the expression pattern [31].

Stress Treatment

Fifteen-day-old homozygous seedlings containing the *Bet v 1a* promoter-*GUS* construct were subjected to various stresses, including incubation at low (4°C) and high (37°C) temperatures, treatments with 0.1 m*M* abscisic acid (ABA) which is a stress- and development-related plant hormone [for a review, see 32], 0.1 *M* NaCl for 12 h and 30% PEG 6000 (simulating drought conditions) for 3 h as described by Ishitani et al. [33]. In order to analyze the putative wound-mediated response of the *Bet v 1a* promoter, wounded vegetative tissues of adult plants as well as parts of flowers were incubated for 16 h in MS medium before examination. To test the effect of temperature and ABA on the activity of the *Bet v 1a* promoter in developing pollen, tobacco pollen culture was employed as described by Touraev and Heberle-Bors [34]. Briefly, isolated microspores of transgenic plants were maturated in vitro at 18°C, 25°C (normal) and 33°C, or in the presence of 0.1 m*M* ABA at 25°C, and cultures were examined either when microspores had completely matured into pollen (in the case of evaluation of the temperature effect) or when microspores had reached the stages of bicellular pollen and mature pollen (to study the ABA-mediated response).

Histochemical and Quantitative Assays

Qualitative (histochemical) and quantitative (fluorimetric) enzymatic analyses of the GUS activity were performed according to Jefferson [35]. The histochemical assay was carried out by staining plant material with 1 m*M* 5-bromo-4-chloro-3-indolyl glucuronide (X-Gluc), which is the indigogenic substrate for β-glucuronidase. During this staining, the indoxyl derivative as the result of the GUS hydrolytic activity forms the blue precipitate at the site where enzyme is located. For the quantitative assay, plant cells/tissues were extracted in GUS extraction buffer and supernatants were incubated with 1 m*M* 4-methylumbelliferyl β-*D*-glucuronide (4-MUG), the GUS fluorogenic substrate. The GUS activity was measured at 37°C as the change in fluorescence of the reaction product 4-methylumbelliferone (4-MU) per minute or per hour (typically, minute intervals for high GUS levels and hour intervals for lower levels of GUS are used) and normalized to the total protein content in the supernatant. The fluorescence was converted to a quantity of 4-MU in nanomoles using the calibration curve. Protein content in milligrams was determined using the Bradford reagent (Bio-Rad, Germany). Nontransformed tobacco and homozygous tobacco plants containing the *GUS* gene under control of the carrot *Dc3* gene promoter [36] served as negative and positive controls, respectively, in both types of analyses. In each case, the experiments were repeated 5 times for the control plants and for each independent transgenic line.

Results

Analysis of a Genomic Sequence Containing the Bet v 1a Promoter

Screening of clones resulted in the isolation of a DNA fragment of 2,327 bp (GenBank Acc. No. AY283055). One terminus of the fragment completely concurred with the 5′-part of the *Bet v 1a* cDNA (GenBank Acc. No. X15877) including the beginning of the translated region. The fragment was reamplified with primers derived from the known originally cloned sequence to examine the fragment integrity. Sequences of both primary and newly synthesized fragments were identical, which means that the originally generated fragment was not a false-positive.

Depending on the distance from a transcription start site (TSS), the terms ‘proximal promoter’ (several hundred nucleotides around a TSS) and ‘distal promoter’ (thousands and more nucleotides upstream of a TSS) are usually used. Both proximal and distal promoters include sets of various elements, which participate in the complex process of regulation of transcription. Most promoter elements regulating transcription are located within the proximal promoter [37]. Therefore, a DNA fragment of

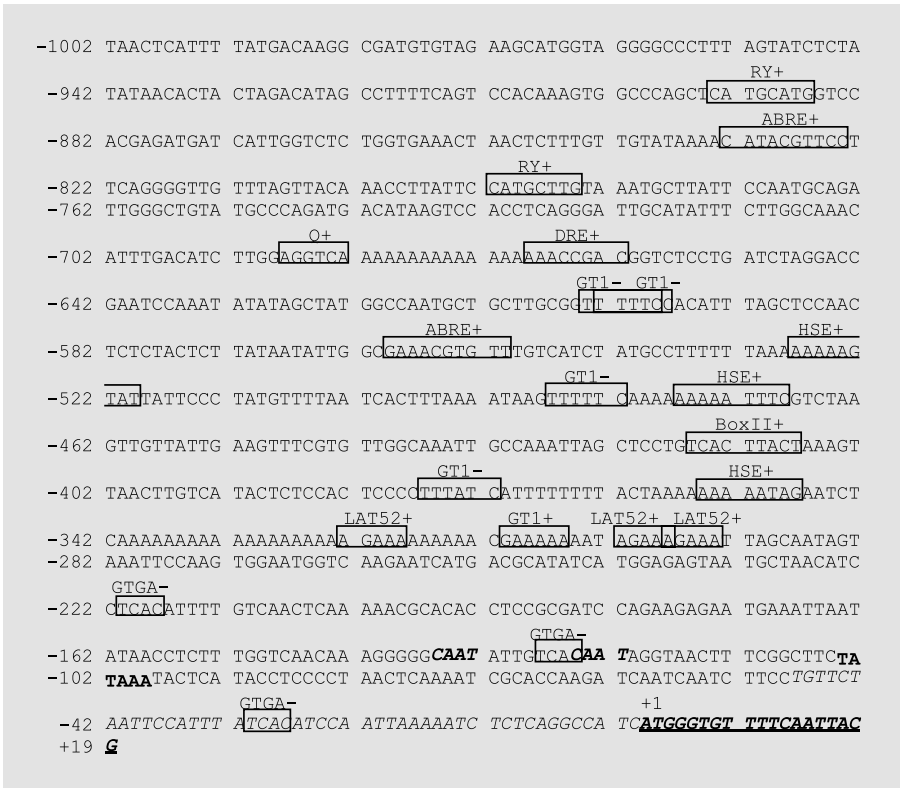

Fig. 1. Sequence of the proximal *Bet v 1a* promoter. The start of translation is marked by +1. Putative CAAT (italic and bold) and TATA boxes (bold), transcribed (italic) and translated (italic, bold, underlined) regions are indicated. Elements that might be involved in pollen-specific expression, ABA- and heat-shock-related induction are boxed; + and –, respectively, designate direct and reverse orientation of the elements. GT1 = GT-1 element; BoxII = Box II element; LAT52 = LAT52 element; GTGA = GTGA element; Q = Q element; ABRE = ABRE element; RY = RY element; DRE = C-repeat DRE; HSE = HSE element.

approximately 1 kb upstream of the transcriptional start site was assumed to contain the proximal promoter region of the *Bet v 1a* gene (fig. 1). Structural analysis of the *Bet v 1a* proximal promoter using network software (see Materials and Methods) identified putative CAAT and TATA boxes and other general regulatory elements modulating gene expression such as Box II [38] and GT-1 [39]. Furthermore, the *Bet v 1a* proximal promoter sequence contains elements responsible for expression in pollen, namely LAT52 [40], GTGA [41], and the enhancing Q (quantitative) element [42] (fig. 1). ABA is known to be a plant stress-related hormone which mediates responses to salinity and drought [32]. The *Bet v 1a* proximal promoter contains three elements shown to mediate ABA induction: ABRE [43], RY element [44] and C-repeat DRE [45] (fig. 1). Further, the *cis*-regulatory motif HSE involved in heat shock response [46] (fig. 1), a wounding-related WUN motif [46] and light-responsive elements AE box [47], GAG motif and LAMP element [38] (data not shown) were predicted.

The distal region of 64 nucleotides at the other end of the cloned fragment showed similarities to the beginning of the translated region of several *Bet v 1* genes like *c, g, k* (GenBank Acc. No. X77265, X77269, X77272, respectively), but in the reverse orientation, and is located 2,244 nucleotides upstream of the ATG codon of the *Bet v 1a* gene. This suggests that the originally cloned fragment contains two promoters in opposite orientations: the *Bet v 1a* promoter and that of a putative gene, we called *Bet v 1x* (GenBank Acc. No. AY283055), which may be an unknown member of the *Bet v 1* family.

Expression Driven by the Bet v 1a Promoter

Since elements within a 'distal promoter' may also modulate transcription [37], the sequence upstream of the proximal promoter might influence the expression of the *Bet v 1a* gene. Therefore, the entire isolated fragment was used to construct the *Bet v 1a* promoter-*GUS* fusion in order to produce an expression profile similar to natural expression. Homozygous F1 transgenic lines were used to determine whether the *Bet v 1a* promoter was able to direct gene expression under normal conditions. Histochemical assays on 13 independent lines did not reveal any positive blue staining, which indicates GUS activity, in vegetative tissues from leaves, stems, roots or in seeds and parts of the flower (data not shown) except in developing pollen. GUS expression was initially weakly visible in late microspores, but then strongly increased through

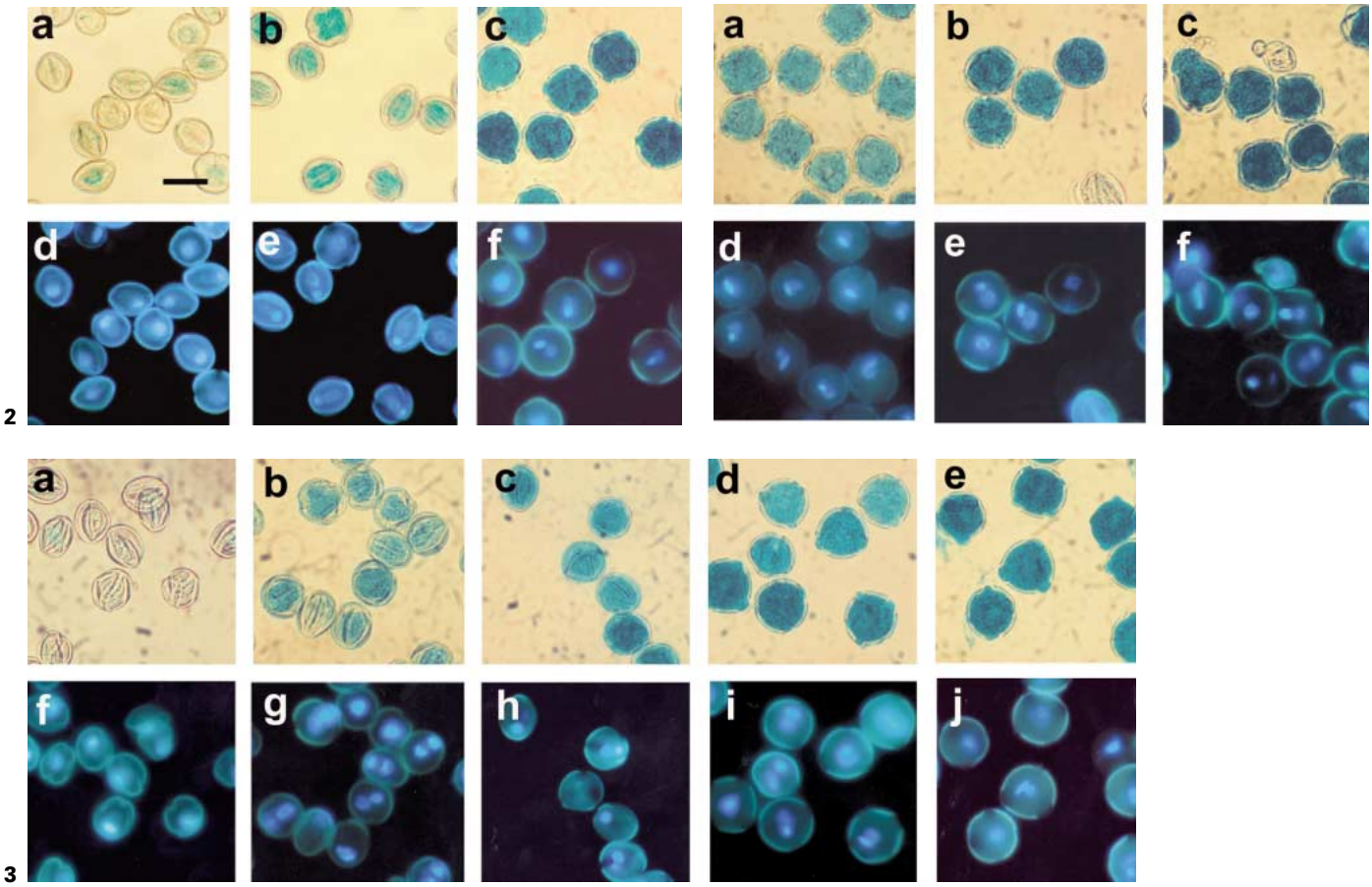

Fig. 2–4. Histochemical analysis of stable GUS expression in tobacco harboring the *Bet v 1a promoter-GUS* construct (transgenic line 1) during pollen development. Cells were stained for GUS expression (upper row of each section) followed by staining with DAPI (lower row of each section) to determine the developmental stage. Images were taken under light (GUS histochemical assay) and fluorescent (DAPI staining) microscopes. Microspores and bicellular pollen were stained overnight, mature pollen during 1 h. **2** GUS expression in cells developed in vivo: late microspores (**a**, **d**), bicellular pollen (**b**, **e**), mature pollen (**c**, **f**). **3** GUS activity determined in developing pollen cultured in vitro in the presence of 0.1 m*M* ABA compared to untreated cells: late microspores (**a**, **f**), bicellular pollen (**b**, **g**), bicellular pollen treated with ABA (**c**, **h**), mature pollen (**d**, **i**), mature pollen treated with ABA (**e**, **j**). **4** GUS expression in mature pollen developed in vitro at 18°C (**a**, **d**), 25°C (**b**, **e**) and 33°C (**c**, **f**). **2–4** Bar: 20 µm.

transition from bicellular to mature pollen (fig. 2). The expression profile from the *Bet v 1a* promoter shared characteristics of so-called 'late' pollen genes, which are activated in association with first pollen mitosis and continue expression as the pollen matures [48]. In contrast, no staining was observed in developing pollen or any other tissue of nontransformed control tobacco plants. Similarly, no stained regions could be observed in the wounded areas of various tissues examined (data not shown).

Five of 13 transgenic lines analyzed histochemically were examined by the fluorimetric GUS assay. Quantitative analysis confirmed the data obtained by the histochemical GUS detection. Seedlings of these *Bet v 1a-GUS* transgenic lines subjected to exogenous ABA, salinity, dehydration (PEG 6000), and low and high temperature stresses did not display any induction of the promoter activity as measured by GUS expression (data not shown). The *Bet v 1a* promoter activity was detected in late microspores and increased 22- to 23-fold in mature pollen. The level of GUS expression mediated by the *Bet v 1a* promoter in pollen was approximately 40% higher than that of the control *Dc3 promoter-GUS* plants (fig. 5a). To the best of our knowledge, the *Bet v 1a* promoter represents the strongest pollen-specific promoter known so far.

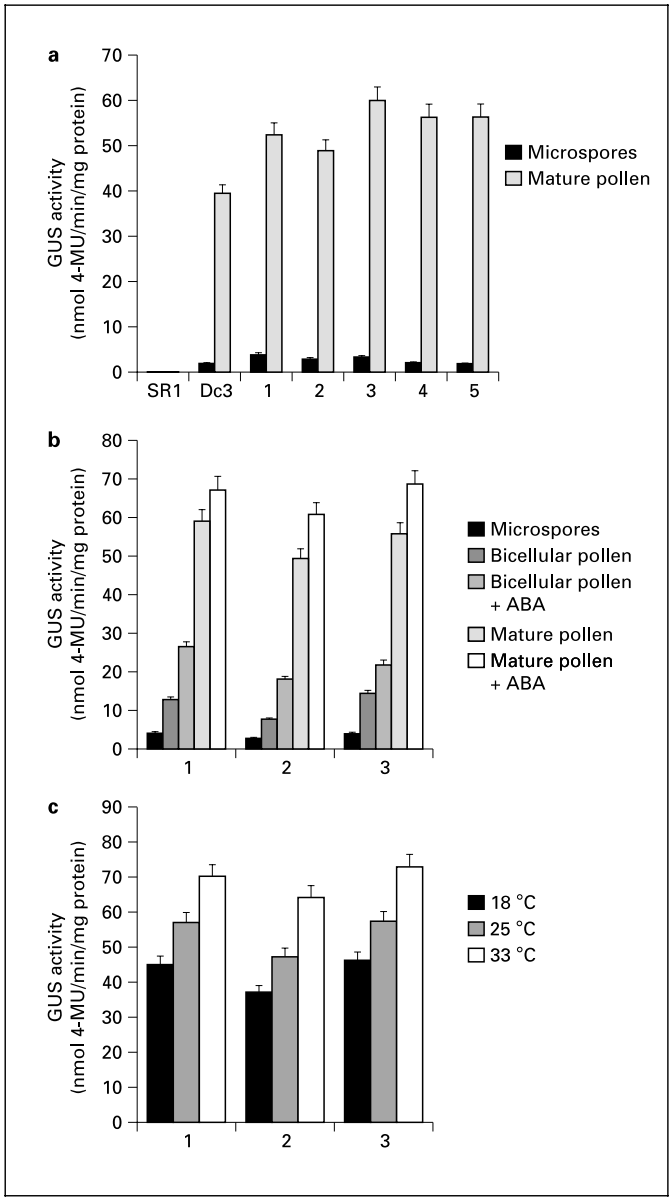

Fig. 5. Fluorimetric assay of GUS activity determined in developing pollen of the *Bet v 1a* promoter-*GUS* transgenic lines. GUS activity was measured as the amount of the reaction product 4-MU in nanomoles formed per minute per milligram of the total protein extract. SR1 = Nontransformed control plant, Dc3 = *Dc3 promoter-GUS* transgene. Numbers designate the independent *Bet v 1a promoter-GUS* lines. **a** GUS activity determined in microspores and mature pollen developed in vivo. **b** GUS expression in in vitro developing pollen treated with 0.1 m*M* ABA compared to untreated cells. **c** Expression of GUS activity in pollen matured in vitro at 18, 25 and 33°C.

To evaluate the effect of ABA and temperature conditions on the induction of the *Bet v 1a* promoter in developing pollen, three transgenic lines were analyzed by both histochemical and fluorimetric assays using in vitro pollen culture. Histochemical staining displayed a much stronger signal in bicellular and mature pollen upon ABA treatment (fig. 3) compared with untreated cells (fig. 2). As measured by fluorimetric assays, the ABA-induced activity of the *Bet v 1a* promoter increased approximately 1.5- to 2-fold in bicellular pollen and 1.2-fold in mature pollen in comparison with untreated cultures (fig. 5b). Temperature positively correlates with the activity of the *Bet v 1a* promoter during pollen development as revealed by the histochemical assay (fig. 4). The quantitative analysis showed about a 25% decrease of GUS activity in mature pollen cultured at 18°C compared to those developed at the normal temperature (25°C). An elevated temperature (33°C) caused an approximately 30% increase in the accumulation of the GUS product compared with control cultures incubated at 25°C (fig. 5c).

Discussion

In this paper we report the creation of a model system to study the role of environmental factors in the induction of the *Bet v 1a* gene, which encodes the major birch pollen allergen. Taking into account the clinical significance of this allergen, the creation of a model system in order to evaluate the environment-dependent induction of the *Bet v 1a* gene under defined laboratory conditions would be highly useful. We think that transgenic tobacco plants harboring the *Bet v 1a* promoter fused to the reporter *GUS* gene could serve as such a model system. Tobacco was chosen to test the *Bet v 1a* promoter activity since a transgenic approach in birch is clearly unrealistic and time-consuming. The term 'model' is used to describe a specified aspect or aspects of a system (http://www.cise.ufl.edu/~fishwick/rube/tutorialsenior /metaphor.htm). The model system created in this work covers one important aspect, namely, the environment-dependent induction of the *Bet v 1a* gene. As a model, this system has limitations and thus does not allow the study of other environment-dependent processes contributing to allergenicity such as allergen accumulation (translation), location and availability of the allergen (posttranslational modifications, protein transport to the pollen surface), or putative proteolytic processing which may increase allergenicity [49, 50]. We are also aware that tobacco is a heterologous expression system. However, the widespread and success-

ful use of tobacco to study the activities of promoters isolated from other species, e.g. carrot, tomato, maize and rapeseed [36, 40, 51–54], convinced us that the proposed model covers the main aspects of the environment-dependent induction of the *Bet v 1a* gene.

In tobacco plants under normal and stress conditions, the *Bet v 1a* promoter activity was shown to be highly restricted to developing pollen and the expression profile was similar to that of 'late' pollen-expressed genes [48]. Strong induction of the *Bet v 1a* promoter in tobacco mature pollen correlates well with earlier observations of Swoboda et al. [55] and Pettenburger et al. [18], who also found an accumulation of the *Bet v 1a* mRNA and protein at the late stage of birch pollen development by in situ hybridization and immunoblotting, respectively. Within the *Bet v 1a* promoter, pollen-specific expression seems to be governed by putative functional modules such as the pollen-specific LAT52 elements in combination with the Box II and GT-1 motifs [56], GTGA elements, and enhanced due to the presence of the Q element (fig. 1). Originally, active GTGA elements were found in direct orientation within the tobacco *g10* gene promoter [41]. Although the *Bet v 1a* promoter contains GTGA elements in the reverse orientation, it might not be essential for the pollen-specific induction since the GTGA element represents a type of Box II core sequence shown to act in an orientation-independent manner [57] as well as GT-1 elements [58].

The presence of the putative ABA-, heat shock- (ABRE, RY, C-repeat DRE, HSE) (fig. 1) and other stress-related elements within the *Bet v 1a* promoter led to the assumption that the activity of the promoter might be dependent on the relevant stress factor. No GUS induction was detected in vegetative tissues of transgenic tobacco seedlings exposed to various stresses. In contrast, exogenous ABA and higher temperature were shown to enhance the transcription by the *Bet v 1a* promoter in developing pollen. These results suggest that environmental factors like temperature or those mediated by ABA affect the activity of the *Bet v 1a* promoter, and that ABA-, temperature-responsive and possibly other stress-related elements within the *Bet v 1a* promoter might interact with tissue-specific transcription factors [32] and direct the expression exclusively to pollen. ABA-mediated regulation of gene expression during late pollen development has been shown to be dependent on desiccation [59]. An ABA-induced shift of the *Bet v 1a* promoter activity to the earlier stage of pollen development suggests that ABA-responsive elements could play a significant role in the activation of the *Bet v 1a* promoter upon pollen desiccation during the transition phase from immature to mature pollen and may mainly contribute to the 'late' character of expression. Future experiments with inhibition of endogenous ABA could confirm this assumption.

The system used in planta in combination with an established in vitro tobacco pollen culture technique [34] will be extremely useful to study directly the effect of environmental factors (such as temperature, drought, air pollution) and ABA on *Bet v 1a* gene expression in pollen. Furthermore, the tobacco expression system makes it possible to evaluate the functionality of the region upstream of the proximal promoter and already predicted promoter elements in the environment-dependent induction of the *Bet v 1a* gene using deletions, gain-of-function analysis and directed mutagenesis in transient or stable transgenic assays. These results may benefit the structural analysis of other plant allergen gene promoters by providing a reference with respect to the functionality of a given motif. Strong expression of the *Bet v 1a* gene during late pollen development is a feature which is similar to other pollen allergens, e.g. profilin [60]. Such similarity might be maintained by the presence of the same mode-responsive elements within the promoters, and thus knowledge regarding *Bet v 1a* gene expression could be applied to that of other pollen genes encoding allergens.

In addition, knowledge regarding the regulation of the *Bet v 1a* gene expression might serve as a prerequisite to focus on a search for compounds preventing gene induction in pollen and thus reduce the potential allergenicity of birch trees.

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