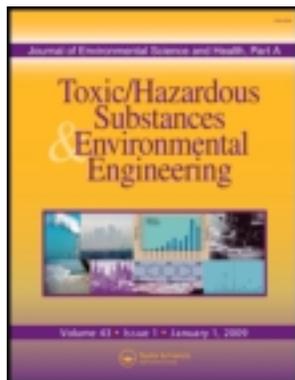


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Changes in expression of BetV1 allergen of silver birch pollen in urbanized area of Ukraine

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The aim of the study was to determinate the level of expression of silver birch allergen Betv1 in pollen samples from different Ukraine areas by RT-qPCR SYBR Green assay. Protocol for quantifying the expression of Betv1 allergen was developed when testing of three housekeeping genes—cyclophylin, alpha-tubulin and transcription factor CBF1. Samples from urbanized area was analysed by real-time PCR when a sample from forest growth conditions was used as a calibrator. Real-time PCR based quantifying of Betv1 provides a useful method for rapid and sensitive analyses of this silver birch allergen. Our results show higher expression levels in samples from central parts of urbanized area as housing estates when compared to the samples from borders of the urbanized area.

Keywords: Quantitation real-time PCR, Betv1 allergen, *Betula pendula* Roth.

Introduction

The prevalence of allergic mainly allergic respiratory diseases has increased in recent years, especially in industrialized countries. This increase may be explained by changes in environmental factors, including indoor and outdoor air pollution.^[1] Hypersensitivity to aeroallergens causes allergic diseases (e.g., bronchial asthma, allergic rhinitis, and allergic conjunctivitis) that affect up to 30% of the population.^[2,3] Therefore, questions about allergy and sources of allergens are often discussed and studied.^[4,5]

When plants are infected by pathogenic bacteria or fungi, injured or attacked by various exogenous stimuli such as air pollution and ultraviolet light, they induce a series of defense responses to protect themselves and produce defense-related proteins.^[6] By attaching pollutants to the surface of pollen grains and of plant-derived particles of paucimicronic size, pollutants could modify not only the morphology of these antigen-carrying agents but also their allergenic potential. In addition, by inducing airway inflammation, which increases airway permeability,

pollutants overcome the mucosal barrier and could be able to “prime” allergen-induced responses.^[1]

Pollen allergens are one of the major sources of respiratory diseases, mainly from grass,^[7,8] but pollen of the European white birch is a major source of spring pollinosis in Europe.^[9] Birch pollen grains can be transported over hundreds or even thousands of kilometers if weather conditions are suitable.^[10]

The major birch pollen allergen is Betv1 protein. The Betv1 protein belongs to the ubiquitous family of pathogenesis-related plant proteins (PR-10), which are produced in defense-response to various pathogens and have been identified in more than 70 species of flowering plants, both mono- and dicotyledons.^[11,12] The 17.5 kDa protein Betv1 is the best known representative of the PR-10 family. It consists of different naturally occurring isoforms, encoded by seven genes, sharing more than 95% sequence.^[13] Among a lot of isoforms of Betv1, 36 of which were annotated to the official list of allergens maintained by the WHO/IUIS Allergen Nomenclature Subcommittee.^[9]

Based on their IgE-binding capacity, Betv1 isoforms were divided into hyper- and hypoallergenic variants. Some of these structurally, closely related proteins are potent allergens, whereas others are less or not.^[14,15] Birch pollen is the mainly source of Betv1 allergen, other parts of birch trees, including other parts of the catkins, express low levels of Betv1.^[16,17] Birch pollen, as a strong allergen is intensively studied: occurrence in atmosphere, quantity and quality,

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broaden and transport to far areas,^[18–21] birch pollen allergen types, isoforms of birch pollen allergen types,^[12,14,15] human patients research.^[22]

Buters et al.^[21] monitored atmospheric concentrations of birch pollen grains and the matched major birch pollen allergen Betv1 simultaneously across Europe within the EU funded project HIALINE (Health Impacts of Airborne Allergen Information Network). The major birch pollen allergen Betv1 was determined with an allergen specific ELISA. The average European allergen release from birch pollen was 3.2 pg Betv1/pollen and the average allergen release in 2009 did not vary substantially between countries. However, a >10-fold difference between daily allergen release per pollen was detected in all countries.

Bryce et al.^[19] showed that birch pollen from urban areas have a higher allergenic potential than pollen from rural areas, although the allergen content remained unchanged. Savitsky et al.^[23] presents the results of aeropalynological observations in Kiev, carried out with a gravimetric method, during January–October, 1994. The six most abundant pollen types were: *Betulaceae* (21%), *Chenopodiaceae/ Amaranthaceae* (10%), *Ambrosia* (10%), *Artemisia* (9%) *Pinaceae* (8%) and *Poaceae* (6%).

Analyse of allergens on the molecular level is made possible by investigation of DNA and real-time PCR is good tool for evaluate of birch pollen allergens. Longhi et al.^[24] used real-time PCR as a rapid, accurate, and automated tool for the detection and quantification of airborne allergenic pollen taxa. Real time PCR is a useful tool for detection: gene expression^[25] identification and quatification of pathogens,^[26–29] authentication of food.^[30] The aim of the study was to determinate relative quantity of birch allergen Betv1 in pollen samples from two Ukraine regions by RT-qPCR.

Materials and methods

Pollen samples

Pollen of *Betula pendula* Roth. has been prepared prior to the beginning of anthesis in Kyiv and Rivne region of Ukraine. Birch catkins were dried at room temperature. After drying, pollen from each catkin was shook off into a plastic aseptic container and stored in a freezer for preventing degradation. Expression level of Betv1 gene was analyzed by using of the quantitative real-time PCR. Silver birch pollen was sampled in within areas as listed in Table 1.

RNA preparation protocol

Total RNA from birch pollen samples was extracted using the GeneJET™ Plant RNA Purification Mini Kit (Fermentas) according to the manufacturer's recommendations and RNA quality and the concentration were quantified spectrophotometrically. Reverse transcription was

Table 1. Pollen samples of *Betula verrucosa* Ehrh, Ukraine.

Sample	Place of growth
1	Kiev (park, housing estate)
2a	Pereyaslav-Khmelnytsky (housing estate, near roads)
2b	Pereyaslav-Khmelnytsky (territory of museum)
3	Hotski (forest)
4	Ivankov (housing estate, near roads)
5	Kuznetsovsk (near forest and roads)
6	Borodyanka (near aeroport)

performed with the Maxima® First Strand cDNA Synthesis Kit for RT-qPCR (Fermentas, Leon-Rot, Germany) using 1000 ng of total RNA extracted from pollen, according to the manufacturer's recommendations and with temperature and time profile listed in protocol.

Real-time PCR procedure and data processing

Primers for real-time PCR were developed on the basis of the mRNA sequences of Betv1 gene and three references genes—alpha-tubulin, cyclophylin and transcription factor CBF1. All of them are listed in NCBI databases under accession number as in Table 2. Primers were designed using Primer-Blast (Bethesda, MD, USA). Primer sequences as used for real-time PCR assays are also listed in Table 2.

To optimize of PCR conditions for each primer, annealing temperature, PCR efficiency standard curve were examined. The specificity of the PCR amplification was checked by end-point PCR and after RT-qPCR it was confirmed by a melt curve analysis.

All reactions were performed in a Biorad CFX96 machine (Bratislava, Slovakia). Primer concentrations were independently optimized to favour product formation then amplification efficiency for each optimised primer pair was calculated using a 2-fold dilution series. Twenty-five microliter reactions containing Maxima® SYBR Green/ROX qPCR Master mix (2X) (Fermentas), 0.2 μM of each primer and 400 ng of transcribed cDNA. Real-time PCR reactions were performed in the volume of 15 μl, used the temperature and time conditions as follows: initial denaturation step at 95°C for 10 min, 40 cycles at 95°C for 15 s, 60°C for 30 s and 72°C for 40 s. Fluorescence was read at 60°C during each cycle.

Melting curves were subsequently determined for each reaction to ensure that single products were produced and the resulting reaction was run on a 1.8% agarose gel to confirm the product was unique and of the correct size. Triplicate preps were subjected to RT-qPCR. Betv1 allergen quantitation was then performed according to the Pfaffl method.^[31] Allergen RT-qPCR results were normalised against the cyclophilin gene and compared to the expression of the same allergen gene in pollen sample 3 (Hotski) that was chosen as the calibrator.

Table 2. The sequences of primers used in this study and their accession codes.

Name of primer	Sequences of primer	Annealing temperature of primer
Tub-Bet-R	CAGAACCAGTGCCTCCACCA	FJ 228477.1
Tub-Bet-F	CTGCCAACAACTTTGCCCGT	
Transcr-Bet-R	AGGGTGGAGGCAAGAGCATC	EF 530204.1
Transcr-Bet-F	TGCGGAGTCTAAGGCTGTGG	
Cycloph-Bet-R	TGCCGGGGCCGGTGTGCTTC	AJ 311666.1
Cycloph-Bet-F	GCCGCTCCGGCAAGCCCCTC	
BetV1-R	CTCCATCAGGGGTTGCCACT	X15877.1
BetV1-F	ATGGAGGGCCTGGAACCATT	

Results and discussion

In the present study, we aimed to develop a real-time PCR method based on SYBR GREEN technology capable of identifying and quantifying a subset of the *Betv1* allergen of birch pollen. Similar approaches based on real-time PCR have already been applied in many different fields to the detection and quantification from transgene constructs^[32] to the allergens contained in foods.^[33]

Selection of a standard comparator gene and validation of RT-qPCR

The ideal control gene should be expressed in an unchanging fashion regardless of experimental conditions, including different tissue or cell types, developmental stage, or sample treatment. Because there is no one gene that meets this criterion for every experimental condition, it is necessary to validate the expression stability of a control gene for the specific requirements of an experiment prior to its use for normalization.^[34]

Cyclophylin, alpha-tubulin, and transcription factor CBF1 were assessed as controls to normalize expres-

sion (accession numbers and the primers are presented in Table 2). cDNA fragments of each gene were isolated by PCR using the primers described and then quantified by comparison with known standards on agarose gels. Ten pMol of each cDNA product was used as a comparator against total cell RNA to estimate relative RNA levels for each gene under the growth conditions described.

Only cyclophylin provided good constitutive controls whereas alpha-tubulin and transcription factor CBF1 was observed to vary considerably in its expression level. As cyclophylin appeared to show a useful constitutive level of expression it was used as a standard in subsequent experiments. Dissociate curves of RT-qPCR amplified products calculated by plotting the negative derivative of fluorescence $[-R'(T)]$ emitted by the PCR sample during the melting procedure (from 65°C to 95°C) showed a single melting peak with melting temperature (T_m) of 80°C for analysed samples and 84.5°C for cyclophylin, indicating specific RT-qPCR product (Fig. 1). Moreover, agarose gel electrophoresis of these products confirmed amplification of a single product and no primer-dimer formation were generated during the RT-qPCR reactions. Control reactions of NTC did not generate any products and no dissociation curves for them were observed.

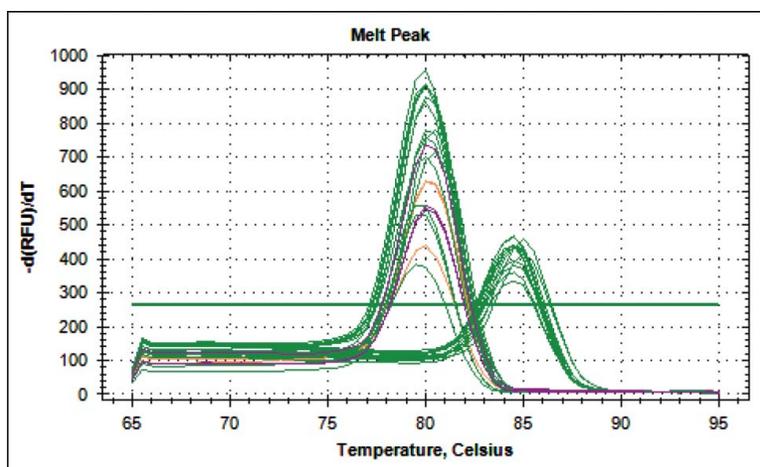


Fig. 1. Melting temperatures of analyzed *BetV1* products and cyclophylin PCR product as visualized by dissociation curves (color figure available online).

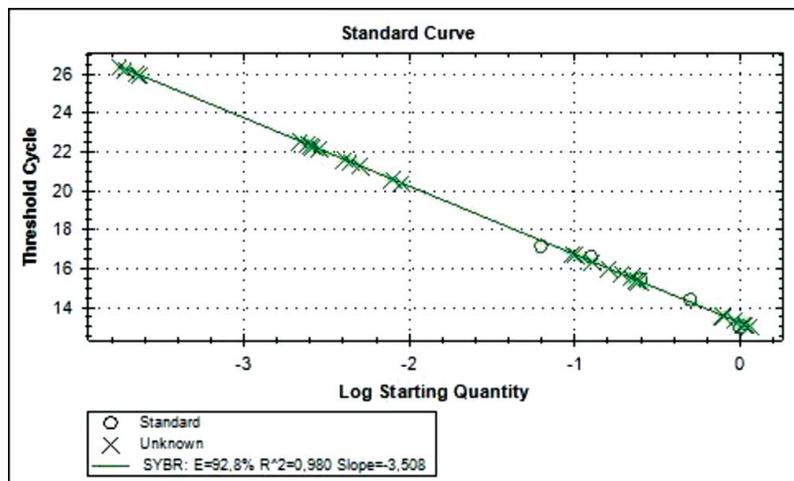


Fig. 2. Standard curve for analysed assays when constructed for cyclophilin gene (color figure available online).

Standard curve and real-time PCR assay

Standard curve was realized by analyzing of 5 serial dilutions of pollen cDNA in 2-fold dilutions achieving the efficiency of 92.8%. Standard curve showed a linear regression between input cDNA and Ct values in the 2 independent assays, with determination coefficients (R^2) of 0.98. Standard curve realized with pollen samples from different places of growth showed good reproducibility, too, because the linear regression analyses demonstrated well-overlapping for the slope values (Fig. 2).

Relative expression of Betv1 allergen

The PR proteins represent an important group of human allergens. Several allergenic PR proteins have been biochemically characterized in detail and that from birch pollen, too.^[3] PR proteins are reported as up-regulated in plants in response to stressors such as drought.

End-point PCR analyses revealed that the Betv1 allergen was transcribed in all birch pollen samples (Fig. 3). The expression of Betv1 allergen gene was analysed upon various growth places around Kiev of tested birch pollen samples. Sample from forest growth condition was chosen as a calibrator for expression analyses. RT-qPCR analyses confirmed the results of the end-point PCR, and enabled a more reliable quantification of Betv1 gene expression levels. RT-qPCR showed a variation in the abundance of allergen transcripts among the samples from different places of growth (Fig. 4).

Buters et al.^[35] monitored simultaneously birch pollen and the major birch pollen allergen Betv1 in different size fractions of ambient air from 2004 till 2007 in Munich, Germany. Differences among levels of expression are well documented for many allergens within different stages of the growth,^[36,37] and studies about different expression lev-

els of allergen for variable climatic conditions exist.^[3] In this study the expression level of Betv1 allergen was proved for six different pollen samples that were collected in urbanized area around Kyiv, Ukraine.

For the correction of an existing sample-to-sample variation, normalization against the control gene was used as both of them, control gene and calibrator served as positive controls. As Figure 3 shows, expression of the Betv1 allergen is much more lower among the birches that grow inside of the urbanized area than those from the marginal areas when nor housing estates nor other types of buildings are in the neighborhood.

These findings are interesting when comparing them to the findings of Bryce et al.^[19] who reported that extracts from pollen collected within the trees in urban areas had higher chemotactic activity on human neutrophils compared to pollen from rural sites, although the allergen content remained unchanged.

Results reported here are fully in concordance with finding below. In samples from urbanized area was the expression of Betv1 allergen in average $1.5 \times$ higher (ranged from 0.77 up to the $2 \times$ higher) when comparing to the forest sample served as a calibrator. In samples from borders of the urbanized area was the expression of Betv1 allergen only $0.55 \times$ higher when comparing to the forest sample.

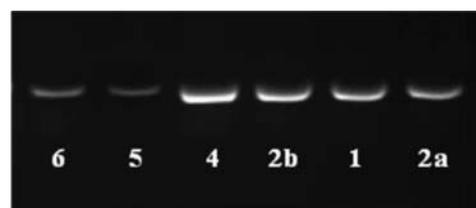


Fig. 3. Specificity checking of Betv1 amplification after RT-qPCR by electrophoresis. Samples codes as listed in Table 1.

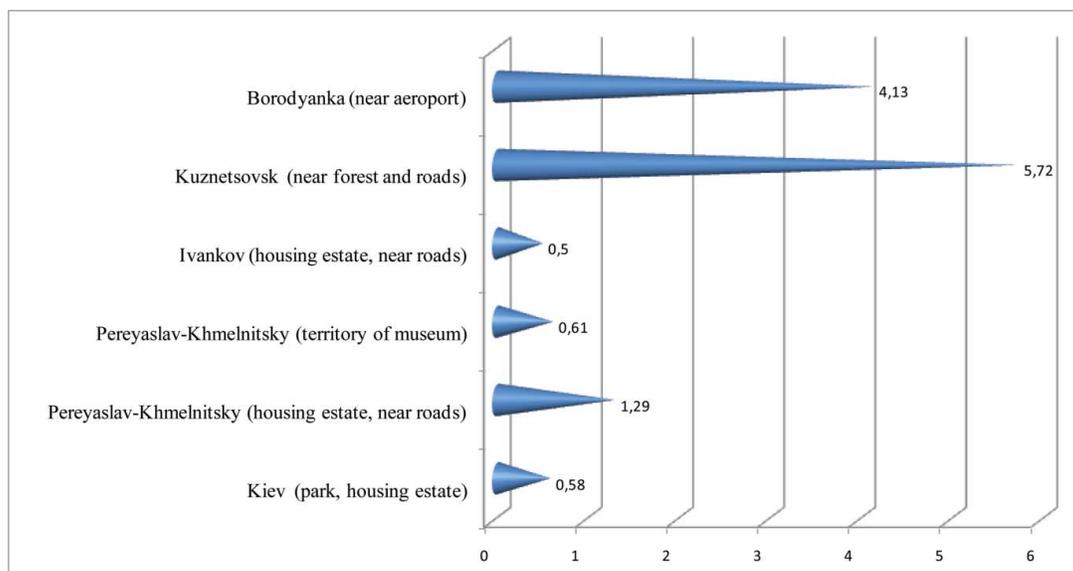


Fig. 4. Visualization of the expression ratios for analysed pollen samples (color figure available online).

Questions about the exact correlations between the expression level and allergenic potential need to be answered in further research. RT-qPCR-based measurement of expression level of Betv1 birch allergen we have developed is in agreement with published data proposing RT-qPCR as a reliable and relatively simple method to infer allergen abundance.^[36,38]

Conclusions

Due to the growing number of allergic reactions, it is crucial to have fast and reliable methods of allergen detection. Until now, no study was realized for comparing the level of the Betv1 allergen expression among Ukrainian birches. In this study was tested three housekeeping genes for a developing of real-time PCR assays targeting Betv1 allergen of birch pollen and the level of the allergen expression was detected for six pollen samples from different areas of growth. The results indicate that PCR assay developed in this study could sensitively detect the changes of the expression levels of the Betv1 allergen and could benefit labs by fast and reproducible method of this allergen detection.

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