

Molecular cloning and characterization of a profilin gene *BnPFN* from *Brassica nigra* that expressing in a pollen-specific manner

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Abstract *Brassica nigra* is a newly found invasive species in Zhejiang Province, China. It distributes alongside the roads, in vegetable fields and on riversides. When it blooms, some natives there will suffer from allergic rhinitis. We designed gene-specific primer pairs according to reported profilin genes and successfully isolated their homolog from flower bud cDNA of *B. nigra*. The gene, designated *BnPFN*, was submitted to GenBank under accession number EU004073. *BnPFN* was 405 bp in length encoding 134 amino acids. Expression analysis of *BnPFN* gene was carried out by means of RT-PCR. The results showed that *BnPFN* express only in anthers and pollens, and there was no detection in roots, leaves, stems, sepals, petals and pistils. We suggest that *BnPFN* is a pollen-specific gene and may be responsible for pollen anaphylactic reactions in those invading areas when *B. nigra* blooms.

Keywords *BnPFN* gene · *Brassica nigra* · Invasive species · Pollen-specific · Profilin

Introduction

Biological invasions are becoming a serious problem nowadays for the introduction of exotic species at a worldwide scale [1, 2]. Introduction of non-indigenous species can either be rewarding or harmful to the ecosystems. Most of the introduced species play important roles as food, herb and ornamental, however, some of them are spreading into the wild where they become invasive and have devastating impacts on native economy and

ecosystems [3–5]. In the United States, the annual economic losses caused by all invasive species, accumulating both their impacts and the costs of responding to them, are estimated at more than US\$ 100 billion [6]. In China, a recent estimate placed its annual economic losses at more than US\$ 14 billion [7]. Besides economic losses, invasive species affect human health. Pollen grains of some Asteraceae, Cruciferae and Pinaceae plants may cause seasonal allergic rhinitis, symptoms predominantly in the nose and eyes in people who are allergic to pollens. As a major family of protein causing allergic reactions to pollen, profilin was first identified from birch (*Betula platyphylla*) [8, 9].

Brassica nigra is an annual weedy plant of family Cruciferae, native to southern Mediterranean region of Europe, and has been cultivated for thousands of years. The plant grows 70–110 cm tall with racemes of small yellow flowers. In recent years, according to our investigation, *B. nigra* is spreading wild in Lishui, Zhuji, Hangzhou of Zhejiang Province, China (unpublished Data). It distributes alongside the roads, in vegetable fields and on riversides. When it blooms, some villagers will suffer from allergic rhinitis. We used gene-specific primers according to reported profilin genes and successfully isolated their homolog from flower bud cDNA and genomic DNA of *B. nigra*, respectively. The gene, designated *BnPFN*, was submitted to GenBank under accession number EU004073. Expression of *BnPFN* gene was carried out by means of reverse transcription polymerase chain reaction (RT-PCR) revealing its pollen-specific expression manner.

Materials and methods

Plant materials

Plant materials were grown in test fields of Lishui University. Fresh roots, stems, leaves, flower buds, flowers,

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sepals, petals, pistils, anthers and pollen grains were harvested and frozen immediately in liquid nitrogen, and then were stored at -80°C for RNA or DNA isolation.

RNA and DNA extraction, cDNA synthesis

Total RNA was extracted from roots, stems, leaves, flower buds, flowers, sepals, petals, pistils and pollens of frozen samples using Trizol reagent (Gibco BRL, Gaithersburg, MD, USA) according to its manual. RNA was treated with RNase-free Dnase I (Promega, Madison, USA) to get rid of DNA. The 1st- and the 2nd-strand cDNA were synthesized using the SMARTTM PCR cDNA synthesis kit (Gibco BRL) according to its instructions. Genomic DNA was isolated from leaves by CTAB method [10].

Isolation of cDNA and genomic DNA sequence of BnPFN

The gene specific primer pairs PFNP1: 5'-ATGTCG TGGCAA(A/G)C(T/A)TA-3' and PFNP2: 5'-CTAGAGA TCCTGTTCGA(G/T)GA-3' were synthesized according to the reported sequences of *B. rapa* and *Arabidopsis thaliana*. cDNA of mixed flower buds (50 ng) and DNA of leaves (80 ng) were used as templates to isolate cDNA and genomic sequences, respectively. In 0.5 ml PCR tubes, 8 pmol of each primer, 150 μM each dNTP, 2 U of *Taq* polymerase (Promega) and 1 $\times$ *Taq* polymerase buffer, and double-distilled H_2O to a total volume of 30 μl were added and mixed. The PCR procedure was initially started with 94°C denaturation for 4 min; followed by 35 cycles of 94°C for 30 s, 52.9°C for 45 s, 72°C for 45 s, and an additional extension for 10 min at 72°C .

The purified PCR products were cloned into the pGEM-T EASY vector (Promega, Southampton, UK) according to its manual. The clones were sequenced on Perkin Elmer Applied Biosystems apparatus 3700.

RT-PCR analysis of BnPFN gene expression

For semi-quantitative RT-PCR, two primers PFNP3: 5'-CG CAATCTTCGGTCAT-3' and PFNP4: 5'-TATGCCTCCT GCTCCC-3' were designed according to the cDNA sequence of *BnPFN* gene. PCR reaction was performed using 50 ng cDNA of leaves, stems, flower buds, flowers, anthers, sepals, petals, pistils, anthers and pollen grains as templates, respectively. In a total volume of 20 μl , 5 pmol of each primer, 150 μM each dNTP, 1.2 U of *Taq* polymerase, 1 $\times$ *Taq* polymerase buffer and appropriate double-distilled H_2O were added and mixed. The PCR procedure was

initially started with 94°C denaturation for 4 min; followed by 33 cycles of 94°C for 30 s, 50.1°C for 30 s, 72°C for 30 s, and an additional extension for 10 min at 72°C .

Results

Isolation and characterization of BnPFN gene

Products of 400 bp and 900 bp were amplified from cDNA and genomic DNA templates, respectively. The full length cDNA sequence was isolated using PFNP1/PFNP2 primer pairs including start codon ATG and stop codon TAG. Sequence analysis showed that the full-size cDNA of *BnPFN* gene was 405 bp in length encoding 134 amino acids (Fig. 1a).

The genomic DNA of *BnPFN* was isolated using the same primer pairs as cloning cDNA sequence. Sequencing results showed that the DNA sequence was 928 bp in length with two introns of 439 bp and 84 bp (Fig. 1b). The deduced protein contains one conserved domain PROF (Fig. 1c) which binds actin monomers, membrane polyphosphoinositides and poly-L-proline [11].

Expression analysis of BnPFN gene

Expression analysis was performed using PFNP3 and PFNP4 primer pairs with equal amounts of cDNA templates prepared from total RNA of different tissues by means of RT-PCR. A fragment size about 200 bp was amplified from flower buds, open flowers, anthers and pollen grains. The expression results indicated that *BnPFN* gene was a pollen-specific one and there were no detection in sepals, petals and pistils (Fig. 2).

Amino acid sequence comparison of profilin genes

For amino acid sequences comparison, we downloaded profilin sequences of 16 other plant materials. These include *A. thaliana*, *B. rapa*, *Nicotiana tabacum*, *Ambrosia artemisiifolia*, *Petroselinum crispum*, *Apium graveolens*, *Daucus carota*, *Zea mays*, *Hevea brasiliensis*, *Phoenix dactylifera*, *Phleum pratense*, *Vitis vinifera*, *Corylus avellana*, *Olea europaea*, *Betula pendula* and *Medicago truncatula*. The deduced amino acid sequences of pollen profilin genes were aligned using ClustalX and GeneDoc softwares (Fig. 3). Phylogenetic tree was constructed based on the alignment of amino acid sequences using NJ methods (Fig. 4).

Sequence comparison revealed high similarity between the deduced amino acid of *BnPFN* and other 16 profilins, indicating their relatively conserved evolution relationship

Fig. 1 Gene structure and deduced amino acid sequence characters of *BnPFN* from *Brassica nigra*. (a) The cDNA and deduced amino acid sequences of *BnPFN* gene; (b) Gene structure of *BnPFN* gene. Solid boxes and lines indicate exons and introns, respectively; (c) Conserved domain of PROF

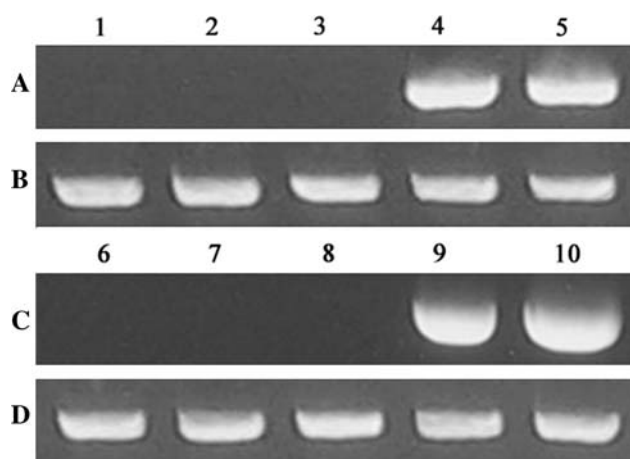
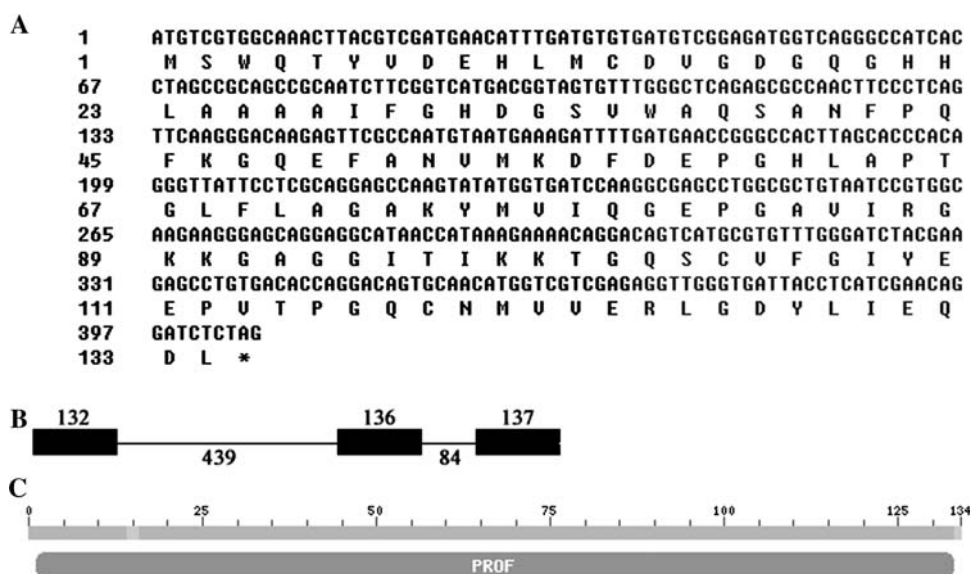


Fig. 2 Expression analysis of *BnPFN* gene from *Brassica nigra*. (a) Expression in different tissues. Lanes 1–5, PCR products of roots, leaves, stems, flower buds and open flowers; (c) Expression in different parts of flower organs. Lanes 6–10, PCR products of sepals, petals, pistils, anthers and pollen grains. (b) and (d) indicate the internal control of actin

under protein level. The phylogenetic tree showed 17 proteins can be divided into two main groups and each group contains several subgroups. *B.nigra*, *B. rapa* and *A. thaliana*, the three Cruciferae plants, are in the same subgroup.

Discussion

Profilins are small and ubiquitous eukaryotic proteins that have been detected in various animal and plant tissues [12–14]. Profilins are best known for interacting with skeleton proteins of the cell, being involved in polymerization of actin filaments and signal transduction events through

phosphoinositide pathways [15]. Profilin can either inhibit or enhance polymerization of actin filaments [16, 17]. Stimulation of actin polymerization will occur while the profilin–actin complex has a lower critical concentration for assembly at the barbed end of the filament when compared with actin alone [18]. However, a potential role of profilin has been identified as a panallergen for its allergenicity [19–22]. The presence of profilin as an allergen was first reported in 1991 [9]. Since then, studies have demonstrated that profilin is an allergen present in a large variety of vegetables, seeds, latex, fruits as well as pollens [23–26].

By far, a great deal of profilin genes had been isolated from various plant tissues. Some of them were pollen-specific and others were constitutive [27, 28]. However, little attention was paid to invasive plants and there were few profilin genes cloned and characterized from them. In our study, we isolated *BnPFN* gene from *B. nigra*, a newly found invasive species in Zhejiang Province, China. The deduced amino acid sequence contains an actin binding site, a phosphatidylinositol-4, 5-bisphosphate domain and a poly-(L-proline) binding site [29].

Expression analysis revealed that *BnPFN* was a pollen-specific gene. The transcript was abundant only in pollens and anthers, and there was no detection in roots, stems, leaves, sepals, petals and pistils. Sequence comparison showed high similarity between the deduced amino acid of *BnPFN* and its homologous allergenic profilins, indicating their relatively conserved evolution relationship under protein level. We suggest that *BnPFN* is the main cause of pollen anaphylactic reactions in those invading areas when *B. nigra* blooms.

Isolation and characterization of the pollen-specific *BnPFN* will be very helpful to understanding allergic reactions caused by its gene product. In our future study,

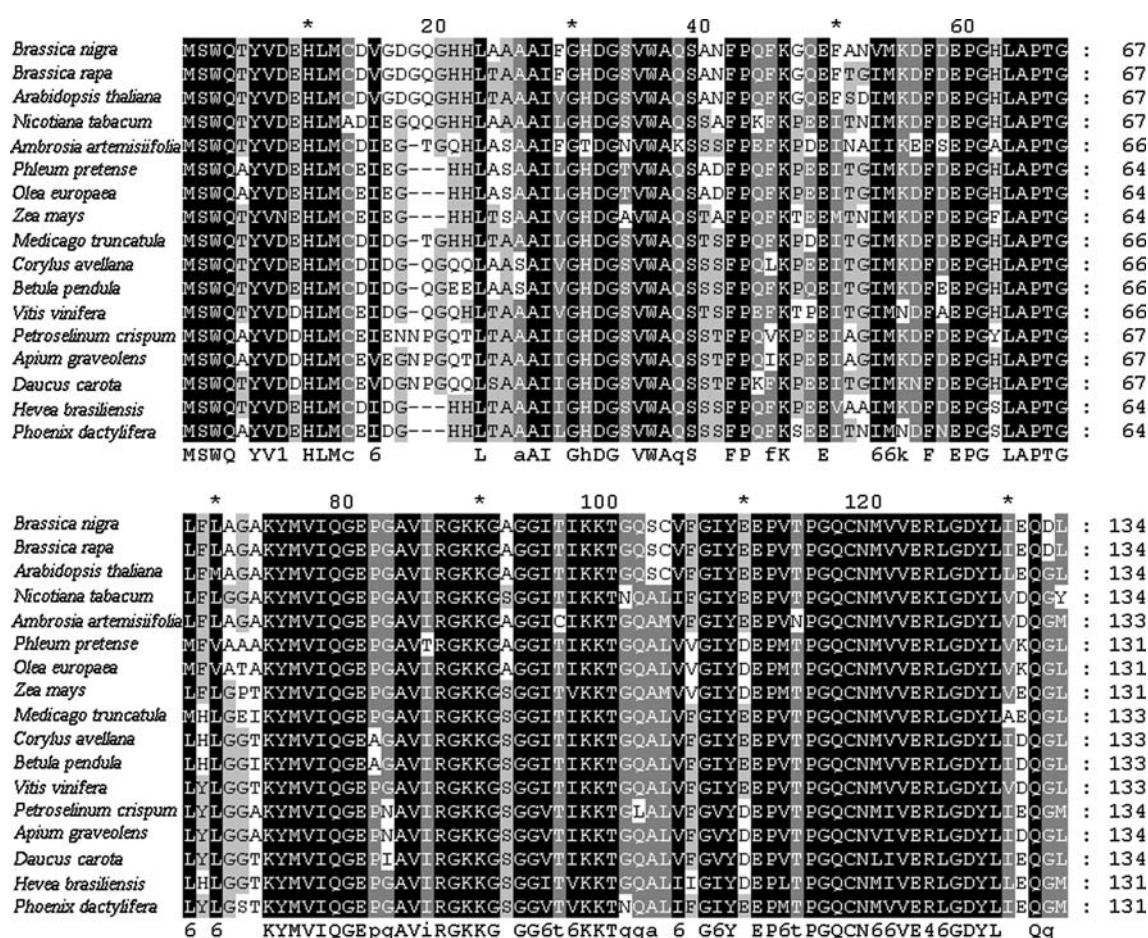


Fig. 3 Alignment of deduced amino acid sequences of *BnPFN* and 16 other profilin proteins using Clustal W. Numbers on the right margin indicate the positions of amino acid residues. The identical amino acids are marked in dark background, and 75% conserved amino acids in gray

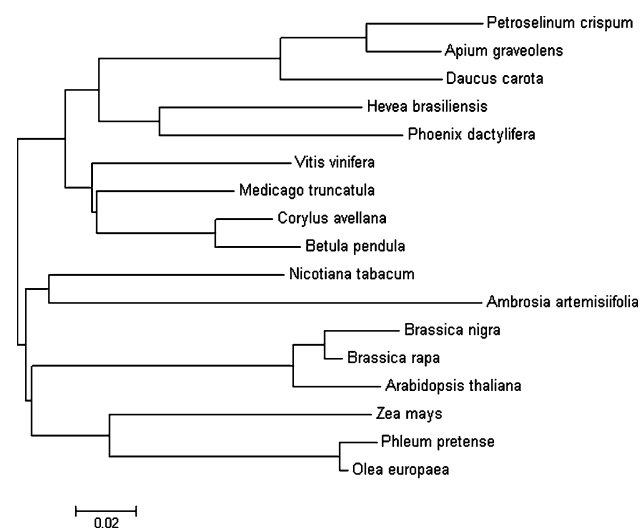


Fig. 4 Phylogenetic tree based on protein sequences of *BnPFN* and 16 other profilins using NJ methods

we will carry out IgE-inhibition and ELISA inhibition experiments to analyze *BnPFN*'s cross-reactivity with profilins.

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