

Class B β -expansins are needed for pollen separation and stigma penetration

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Received: 10 February 2009 / Accepted: 14 May 2009 / Published online: 4 June 2009
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Abstract Group 1 grass pollen allergens comprise a distinctive clade within the β -expansin family of cell wall-loosening proteins and are divided by sequence divergence into two phylogenetically separable classes (A and B). They have been proposed to loosen the walls of the stigma and style. Supporting this idea, we recently showed that a transposon insertion in one of the maize group-1 allergen genes reduces the ability of pollen to effect fertilization under conditions of pollen competition. In this work, we provide additional information on the phenotype of this

mutant, showing that pollen deficient in β -expansin gene expression tended to form large aggregates, leading to poor pollen dispersal on anther dehiscence, and that emerging pollen tubes had difficulties entering the silk. In addition, a silencing construct was created to reduce expression of all the class B genes with results that are consistent with those seen with the transposon insertional line, including reduced transgene transmission through the pollen. Our results provide a more detailed understanding of the role of group 1 allergens (pollen β -expansins) in maize pollen development, pollen dispersal, pollen tube penetration into the style, and pollen tube growth through the transmitting tract.

Communicated by Scott Russell.

Electronic supplementary material The online version of this article (doi:10.1007/s00497-009-0099-y) contains supplementary material, which is available to authorized users.

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Keywords Beta-expansins · Pollen · Pollen allergens ·
Pollen tube · *Zea mays*

Introduction

Grasses (Poaceae) rely on wind for pollen dispersal, producing vast amounts of pollen and coincidentally creating a common human health problem. Approximately 25% of the human population suffers from allergies, and grass pollen ranks among the main contributors (Valenta et al. 1991; Casolaro et al. 1996). Although much is known about the structure, and biological and immunological properties of the pollen proteins responsible for pollen allergies (Shcherban et al. 1995; Li et al. 2003; Yennawar et al. 2006; Griffith et al. 1991; Knox et al. 1993; Laffer et al. 1996), far less is known about their function in the development and performance of the pollen.

When grass pollen are released from the anther, they are metabolically quiescent and dehydrated, with water content ranging from 15 to 35% (Buitink et al. 2000; Heslop-Harrison 1979). When pollen grains land on a receptive dry

stigma, the lipid-rich pollen coat forms an interface between the pollen and the stigma. Water, nutrients and other small molecules are then transported very rapidly into the pollen grain. The mechanisms involved in this transport remain unknown (Edlund et al. 2004). After hydration, the pollen grain germinates, producing a pollen tube that enters the stigma and grows intercellularly toward the transmitting tract. Once in the transmitting tract, the pollen tube grows through the style until it reaches the ovary and double fertilization occurs (Dellaporta and Calderon-Urrea 1994; Freeling and Walbot 1994). The growth of a pollen tube is rapid in maize. Pollen germination typically occurs within 5 min of deposition on the silk, and the pollen tube grows through as much as 50 cm of silk at the remarkable rate of 1 cm/h (Bedinger 1992).

In maize, the proteins associated with the pollen coat include hydrolases and regulating proteins synthesized inside the pollen and secreted before, during or immediately after germination (Cosgrove et al. 1997; Taylor and Hepler 1997; Knox 1984). Among those proteins are group 1 pollen allergens, a group of closely related antigenic proteins, collectively known as Zea m1 (Broadwater et al. 1993). Grass pollen allergens have been classified into 13 distinct groups based on their structure, and biological and immunological properties. Group 1 allergens are one of the most potent allergens and their protein sequence was found to be homologous to that of expansins, a group of cell wall proteins that induce wall loosening. Group 1 allergens from maize were purified and shown to have cell wall loosening characteristics and were thus considered a new class of expansin, currently grouped within the EXPB or β -expansin family (Cosgrove et al. 1997; Sampedro and Cosgrove 2005). We will refer to the group 1 pollen allergens as pollen β -expansins. Based on these and other results, it was hypothesized that pollen β -expansins aid in the penetration of the pollen tubes into the grass stigma and style and facilitate their passage through the transmitting tract (Li et al. 2003; Yennawar et al. 2006).

Pollen β -expansins purified from maize bind to arabinoxylans, which are the major noncellulosic polysaccharide in grass primary walls (Yennawar et al. 2006). Although the details are not clear, arabinoxylans can bind to cellulose microfibrils through hydrogen bonds and may connect microfibrils to each other, either directly or through intermediary polysaccharides (Carpita and Gibeault 1993). The action of pollen β -expansins is proposed to involve binding to arabinoxylans, transiently releasing them from the cellulose microfibrils when the wall is under tension, thus allowing the microfibrils to move apart from each other (Yennawar et al. 2006).

In a recent study examining the activity of maize pollen β -expansins, four isoforms, named Zea m1a,..., Zea m1d, were purified and characterized (Li et al. 2003). The four

glycoproteins are basic (pI of 9.1–9.5) and 27–28 kDa in size. Each isoform induced rapid wall extension, enhanced wall stress relaxation and was selective for grass cell walls. This study also identified four cDNAs, each coding for one of the isoforms. In a more recent analysis, 15 different pollen β -expansin genes were found in the genome of maize cultivar B73, many of them coding for identical or nearly identical proteins (Valdivia et al. 2007a). According to these results, the most abundant isoform, Zm1d, is encoded by three highly similar genes in cultivar B73: *EXPB1a*, *EXPB1b* and *EXPB1c*.

On the basis of sequence similarity, pollen β -expansins have been divided into two classes, A and B, both of which have been identified in all grass species for which there is enough sequence information (Li et al. 2003). Of the 15 β -expansin genes in maize cultivar B73, 11 are in class A and 4 in class B (including three coding for Zm1d). The class A genes can be separated into two divergent branches, but all the class B genes are at least 98% identical (Valdivia et al. 2007a).

The expression of class B pollen β -expansins is developmentally regulated, occurring at low levels prior to the first pollen mitosis and at high levels after the second mitotic division (Broadwater et al. 1993). The highest levels of allergens occur in mature pollen (Knox and Suphioglu 1996a; Valdivia et al. 2007a). Allergen genes in grasses are probably expressed late in pollen development, coincident with the accumulation of proteins and carbohydrates (Knox and Suphioglu 1996b). Group 1 pollen allergens have been shown to move to the surface of the pollen grain through the cell wall when the pollen is moistened (Howlett et al. 1981). In a study examining the localization of Zea m1, Wang et al. (2006) discovered that a small amount of Zea m1 exists in the pollen coat fraction of mature pollen grains collected at anthesis. Zea m1 was also localized to the outer pollen wall (tectum and the foot layer of the exine). A substantial amount of Zea m1 was also found in the pollen interior.

We have previously shown that the presence of a transposon insertion in one of the maize pollen β -expansin genes (*EXPB1*) reduces the competitive ability of pollen *in vivo*, due to slower penetration of the pollen tube through the stigma and slower growth within the style (Valdivia et al. 2007b). Using the Pioneer Hi-Bred TUSC collection (Meeley and Briggs 1995), a single line with a *Mu* transposon insertion in *EXPB1* was identified (Valdivia et al. 2007b). Analysis of protein extracts revealed that the overall Zea m1 production was reduced in *expb1* pollen compared with the overall Zea m1 production in *EXPB1* pollen. Zea m1 production was not completely eliminated because, as discussed above, multiple genes contribute to the Zea m1 pool (Valdivia et al. 2007a). Our results also showed that slower pollen tube growth, by delaying silk senescence, increases the risk of fungal infestation (Valdivia et al. 2006).

In the present work, we provide additional information on the phenotype of the transposon insertion line, which showcases the significance of pollen β -expansins in maize reproduction. These proteins seem to be critical for proper pollen maturation and the initial penetration of the silk. In addition, a silencing construct was created to downregulate all the class B genes, with results that confirm and extend with those seen with the transposon insertion line, supporting the functions proposed for pollen β -expansins and adding some new functions.

Materials and methods

Plant material

From a large library of maize lines bearing Robertson's Mutator (*Mu*) insertions (obtained from Pioneer Hi-Bred International Inc., Johnson, IA, USA; see Meeley and Briggs 1995; Benson et al. 1995; Brutnell 2002), we identified a single line with a *Mu* insertion in *EXPB1*. From this line, we created, via repeated backcrosses into *EXPB1/EXPB1* siblings for three generations and then self pollinations, true breeding mutant plants (*expb1/expb1*) and wild-type plants (*EXPB1/EXPB1*), as well as heterozygous (*EXPB1/expb1*) plants by crossing the true breeding plants under standard greenhouse conditions and normal field conditions (Valdivia et al. 2007b). Maize plants with a *Mu* insertion in *EXPB1* were identified through PCR genotyping and by crossing to wild type (*EXPB1/EXPB1*). A total of four *expb1/expb1* F2 plants were identified. To avoid pollen contamination from neighboring plants, controlled pollinations were performed. Ears were bagged before silk emergence. The tip of the husks and the silks were cut off when silks were first visible. Fresh pollen was collected at anthesis. Anthers and debris were removed by filtering pollen through a sieve. Silks were pollinated 1–2 days after cutting, when silk length reached 1.5–2 cm.

In order to compare pollen development, pollen morphology, pollen viability, pollen germination in vitro pollen tube growth and *EXPB1* expression, leaf tissue was collected at the six-leaf stage, anthers were collected 1–3 days prior to anthesis, and mature pollen was collected at the shedding stage from true breeding mutant plants (*expb1/expb1*), wild-type plants (*EXPB1/EXPB1*), heterozygous (*EXPB1/expb1*) plants and plants containing an RNAi construct for β -expansins (see below). Materials were used directly for analysis or frozen in liquid N₂ and stored at –80°C.

Pollen viability, germination and tube growth

In order to determine the viability of pollen from the three types of plants, 25 mL of fresh pollen was placed in a

1.5 mL microcentrifuge tube before adding 750 μ L of a solution of 1% thiazolyl blue tetrazolium bromide (MTT) in 5% sucrose. MTT is modified by a cellular dehydrogenase, with the result that viable pollen stain purple. The tubes containing pollen were incubated at room temperature for 20 min, then placed on ice. Pollen was examined under a light microscope and was considered viable if it turned a deep purple (Rodriguez-Riano and Dafni 2000).

Separation of fertile pollen from sterile pollen from each of the three types of plants was performed following a protocol modified from Wang et al. (2004). Anthers from maize plants were fixed in Farmers solution (3:1 ethanol: acetic acid) for 2 h, followed by 70% ethanol also for 2 h. The fixed anthers were cut in half longitudinally and placed in a solution of 10% mannitol in 0.1 M Pipes (pH 6.9). Anthers were gently shaken in the medium for 15 min. After removing the floating anthers with tweezers, the pollen suspension (1 mL) was loaded on top of 4 mL of a 42% Percoll (Sigma) solution placed in a glass culture tube (10 mL). Sterile pollen should form a distinct layer between the suspension and the Percoll solution; fertile pollen should settle to the bottom of the tube. After 10 min, 750 μ L was removed from three layers: A (top, corresponding to the medium above the Percoll), B (middle), C (bottom). The absorbance of the different aliquots was measured at 400 nm. To compensate for different amounts of pollen, the absorbance of the three regions for each pollen sample was normalized by dividing by the sum of the three absorbance values.

To compare pollen germination efficiencies and performance of pollen from plants with the *Mu* insertion to that of pollen from wild type, an in vitro pollen tube growth assay was performed. As much as 50 μ L of pollen was added to a 1.5 mL microcentrifuge tube. To each tube, 1 mL of pollen germination media (PGM) (0.01% H₃BO₃, 10 mM CaCl₂, 0.05 mM KH₂PO₄, 0.1% yeast extract, 10% sucrose, 6% PEG 4000) (Schreiber and Dresselhaus 2003) was then added. Two 500 μ L aliquots of the pollen solution were then spread on plates containing a solid germination media (PGM, 1% Nobel agar). The pollen was distributed evenly and the excess liquid removed with a pipette. Plates remained at room temperature for 20 min. Pollen tube growth was stopped by spraying plates with 70% ethanol. The field of view of the microscope was centered on each plate and we recorded the lengths of the first 20 tubes encountered on a left to right transect. The pollen tubes were measured using image analysis as described in Rich et al. (1989).

In order to visualize pollen penetration and growth through the silks, pollen collected as described above was used to pollinate silks from *EXPB1/EXPB1* plants. Pollen was allowed to germinate and grow through the silks for 4 h after pollination. A 6 cm region of silk (from the top of

the exposed silk to 6 cm down) was excised. The tissue samples were placed in scintillation vials containing FAA (3.7% formaldehyde, 5% acetic acid, 50% ethanol, 41.3% water). The tissue remained in FAA overnight at room temperature. The tissue was then rinsed in 100% ethanol and hydrated through a series of 10 min washes (75, 50, 30 and 0% ethanol). The samples were cleared in 8 N NaOH for 45 min, rinsed three times in dH₂O and then allowed to remain overnight at 4°C in dH₂O to get rid of any trace of NaOH. The tissue was finally stained with a solution of 0.1% aniline blue in 0.1 N K₃PO₄ for 30 min. The silks and ovaries were then placed on microscope slides and observed under UV light.

Imaging of pollen grains for pollen viability and pollen tube growth studies were performed on a Zeiss Axioplan (Opto-systems Inc, Jenkintown, PA). Digital images from these studies were obtained using a ProgRes C14 camera and software (JENOPTIK Laser System GmbH, Munich, Germany).

Quantitative RT-PCR

To examine the expression of *EXPB1* in pollen from *EXPB1/EXPB1* and *expb1/expb1* plants, RT-PCR assays were performed using total RNA isolated from mature maize pollen. cDNA was prepared using the ABI High Capacity RT kit (Applied Biosystems, Foster City, CA) and was amplified and quantified on the ABI 7300 Real-Time PCR System (Applied Biosystems). Thermal cycling conditions were as follows: 2 min at 50°C, 10 min at 95°C followed by 40 cycles of 15 s at 95°C and 1 min at 60°C. Relative abundances of each gene were determined using the $\Delta\Delta C_T$ method (Applied Biosystems) using cytosolic glyceraldehyde-3-phosphate dehydrogenase (GADPH) for normalization. Primers and probes were designed using Primer Express 1.0 (Applied Biosystems). Primers were synthesized on the Bioautomation MerMade 12 (Plano, TX) at the Nucleic Acid Facility (Huck Institute, The Pennsylvania State University). Probes were synthesized by Biosearch Technologies (Novato, CA) and labeled with the reporter dye 6-carboxyfluorescein (6'-FAM) at the 5' end and with the quencher dye Black Hole Quencher at the 3' end. Primers and probes are summarized in Supplementary Table 1 in the supplementary materials.

RNAi constructs and microprojectile bombardment

To corroborate the results of the *expb1* lines with decreased expansin levels (Valdivia et al. 2007b), we needed to further reduce or knock out the expression of *EXPB1*. A silencing construct was created using a 399 bp DNA fragment from the coding region of *EXPB1*, amplified using primers j39sen-1 (5'-CAGAAGGGTGCGATGCAAG-3') and j39sen-2

(5'-TTAATTAGCTTGCTGCTGAA-3'). The sense fragment was first cloned with TOPO TA Cloning Kit (Invitrogen, Carlsbad, CA) and then introduced into the pUC18 vector. The pollen-specific promoter (1,507-bp) of a rice β -expansin gene, *Ory s1*, also known as *OsEXPB1*, was used to drive the construct (Smith et al. 2000; Wesley et al. 2001). This promoter was fused with the 399 bp fragment from the coding region of *EXPB1* (AY197353). This “sense” fragment was followed by the third intron of *EXPB1* (99 bp) and the “sense” gene fragment in antisense orientation. The construct was completed with the nopaline synthase (NOS) terminator (Depicker et al. 1982).

Iowa State University Plant Transformation Facility (ISUPTF) (Wang 2005) performed microprojectile bombardment using embryonic callus from HiII (A188 X B73) as described in Frame et al. (2000, 2002). A plasmid containing the *bar* gene (bialaphos resistance) driven by a ubiquitin promoter was co-delivered in order to select for transformants on bialaphos (DeBlock et al. 1987). Reciprocal crosses were performed between the regenerated transgenic plants (T₀) and wild-type (inbred B73) plants when possible. The seeds produced from these crosses were referred to as T₁. T₁ seeds were grown and reciprocal crosses with wild-type plants were performed when possible. Seeds produced were referred to as T₂.

DNA extraction and PCR genotyping

A total of 42 callus samples were analyzed for the presence of the transgene. Callus (0.1 g) was placed in a microcentrifuge tube and ground using a disposable plastic pestle (Kimble). Immediately, 400 μ L of extraction buffer [200 mM Tris-ClH (pH 7.5), 250 mM NaCl, 25 mM EDTA, 0.5% SDS] was added, followed by 400 μ L of saturated phenol. Tubes were centrifuged for 10 min at 12,000 rpm at 4°C, and 380 μ L of the aqueous phase was pipetted into a clean Eppendorf tube. To this sample, 400 μ L of chloroform was added. The tubes were then spun for 10 min at 12,000 rpm at 4°C. Approximately 350 μ L of the aqueous phase was pipetted out and placed in a clean Eppendorf tube, 300 μ L of isopropanol was added and the mixture was spun at 12,000 rpm for 10 min. The isopropanol was decanted and the pellets allowed to air dry for 15–30 min. The pellet was resuspended in 50 μ L of water. To check DNA quality, 2 μ L of this extract was separated on a 1% agarose gel.

To identify maize plants that were regenerated from the calli containing the transgene insertion, DNA was extracted from leaves using a rapid prep protocol (Vejlupkova and Fowler 2003) or a modified CTAB method (Sagai-Maroo et al. 1984). PCR was performed with primers Prom-L (5'-GGTCAGCCCGACAAAGTAGGTAGC-3') and Intron-R

(5'-CAGTTAGCTAGATATGTCCATGCATGCAAAC-3'). PCR conditions were as follows: 94°C for 2 min, followed by 30 cycles of 94°C for 15 s, 62°C for 30 s and 72°C for 2 min, finishing with 72°C for 10 min.

Transgenic T_1 and T_2 plants were initially identified by testing for bialaphos resistance. To test for bialaphos resistance, a 2.5 cm × 5 cm region of leaf was delimited with a permanent marker. The leaf surface was then treated with 1.5% Liberty (commercial name of bialaphos, Agro-Evo, Wilmington, DE), which was applied with a sponge. The marked area was scored visually for the presence or absence of necrosis 4–5 days later (Raizada and Walbot 2005). Plants that were resistant to bialaphos were then screened by PCR (see above) to confirm the presence of the transgene.

RNA blot

Total RNA was extracted from mature pollen from plants containing a RNAi construct and wild-type plants, with Concert RNA (Invitrogen, Carlsbad, California) according to the manufacturer's instructions. As much as 8 mg of total RNA was separated on a 1% denatured agarose gel (6.5% formaldehyde) and transferred using Vacugene XI (Pharmacia Biotech, Amersham Pharmacia, Piscataway, NJ) to a Hybond-N nylon membrane (Amersham Biosciences, Piscataway, NJ). The membrane was pre-hybridized using Ultrahyb solution (Ambion, Austin, TX) overnight at 37°C. It was then hybridized to the probe in the same solution at 62°C for 6 h. The blots were washed three times at 60°C for 20 min, twice in 5× SEN (5% SDS, 1 mM EDTA, 40 mM Na₂HPO₄) and twice again for 20 min in 1× SEN (1% SDS, 1 mM EDTA, 40 mM Na₂HPO₄). Blots were then exposed to phosphor screens (Molecular Dynamics, Sunnyvale, CA) for 4 h at room temperature.

The sense fragments of the silencing constructs were used as probes. All probes were labeled with ³²P-dCTP using Rediprime II random prime labeling system (Amersham Pharmacia, Piscataway, NJ). PCR conditions were as follows: 94°C for 2 min, followed by 30 cycles of 94°C for 15 s, 56°C for 30 s, 72°C for 2 min, finishing with 72°C for 10 min.

Results

Pollen phenotype of *Mu* insertional lines

Pollen aggregation

Pollen bearing the *Mu* insertion (*expb1*) tended to be shed in clumps, making distinguishing and separating individual pollen grains difficult (Fig. 1a), whereas wild-type pollen

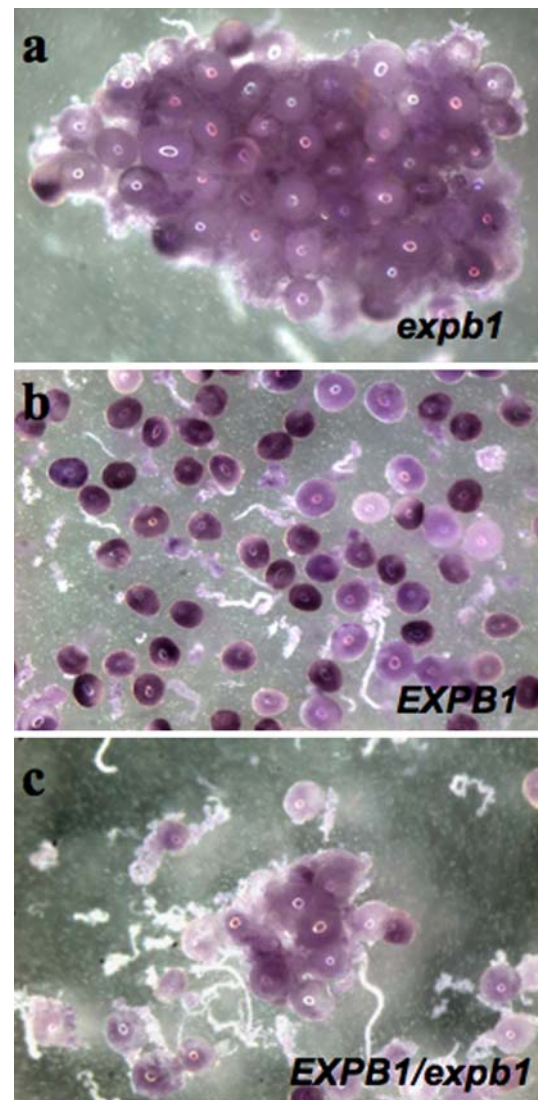
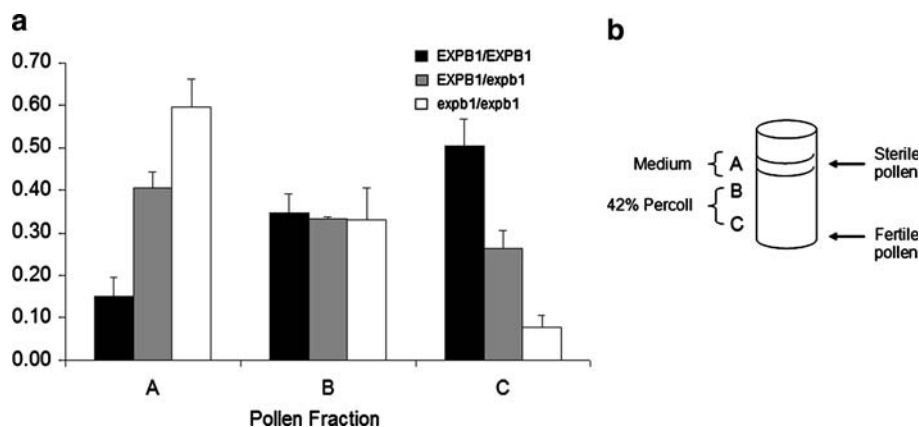


Fig. 1 Pollen aggregation. **a** *expb1* pollen stained with MTT. **b** *EXPB1* pollen stained with MTT. **c** Pollen from *EXPB1/expb1* plant stained with MTT

separated readily from each other. Pollen aggregates were observed in pollen from *EXPB1/expb1* plants; however, the aggregates contained few pollen grains and non-clumping pollen was also present (Fig. 1c). Pollen aggregation was occasionally seen in pollen from wild-type plants; e.g., in field experiments that were performed under high humidity. Especially after rainfall, pollen from wild-type was also sticky and formed aggregates. However, in the case of *Mu* insertional lines, the pollen formed aggregates even during dry conditions.

To characterize the pollen aggregates further, we adapted a method involving centrifugation of pollen into a 42% Percoll layer (Wang et al. 2004). This method, which was originally developed to separate low-density sterile pollen from high-density fertile grains, readily discriminated

Fig. 2 Pollen sedimentation analyses. **a** Absorbance of different pollen fractions. Error bars indicate standard deviation ($N = 4–5$). **b** Diagram showing layers after separation



among pollen from *EXPB1/EXPB1*, *EXPB1/expb1* and *expb1/expb1* plants (Fig. 2). A much larger proportion of pollen from *expb1/expb1* plants remained in the top layer, where sterile pollen would be found. However, pollen from *expb1/expb1* plants has nearly the same viability as that of wild-type plants (Valdivia et al. 2007b). It seems likely that the pollen from *expb1/expb1* plants remained in the top layer due to entrapment of liquid or air within the pollen aggregates, reducing their density. The results for pollen from *EXPB1/expb1* plants were intermediate between those of wild type and *expb1/expb1*, suggesting that the sticky pollen phenotype is restricted to pollen bearing the *Mu* insertion (*expb1*).

In two separate plantings in the greenhouse, the pollen from *expb1/expb1* plants were not shed. Examination of anthers from *expb1/expb1* plants 2 days before anthesis revealed normal pollen. However, at the time when anthers emerged from the spikelet, the pollen failed to shed. When anthers were examined 24 h after they first emerged, the pollen appeared to have dried up. It is likely that the failure of the pollen to shed was due to the clumping of the pollen grains inside the anther.

Pollen tube penetration into the silk

Pollen bearing the *Mu* insertion (*expb1*) exhibited visible differences in their germination and tube growth on the stigmatic surface of *EXPB1* silks, as compared to pollen from wild-type plants (Fig. 3). The *expb1* pollen tubes frequently twisted and wound around the receptive silk hair before penetrating the silk, whereas the wild-type pollen promptly penetrated the stigmatic hairs. Additionally, pollen tubes from *expb1*-bearing plants often attempted to enter the silk surface directly rather than through the silk hairs, which is the normal route of entry (Fig. 3a).

To examine the pollen tube penetration defects in more detail, pollen samples from ten *EXPB1/expb1*, ten *expb1/expb1* and eight *EXPB1/EXPB1* plants were collected and used to pollinate *EXPB1* silks. For each pollination, two to

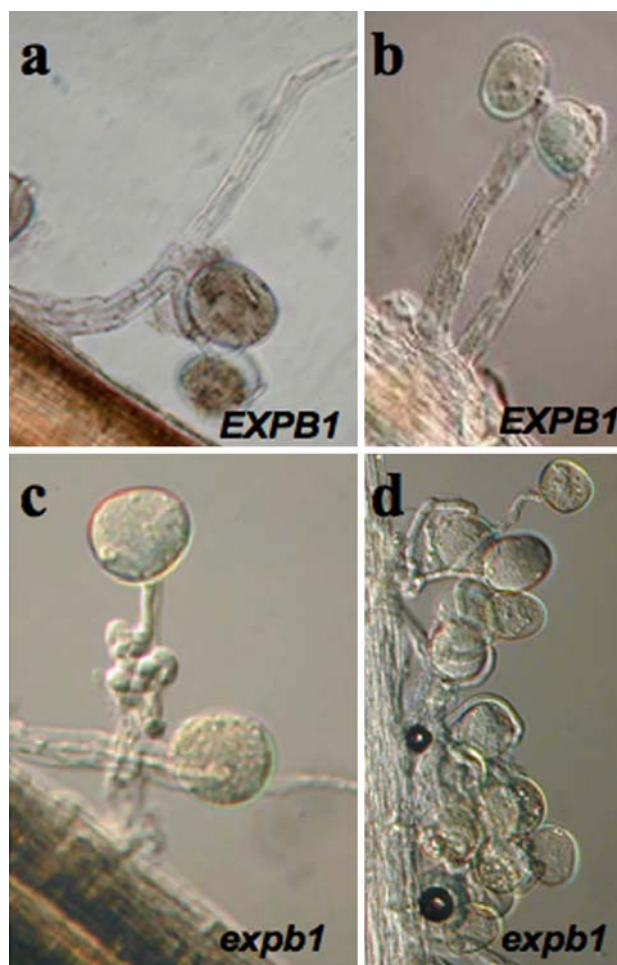


Fig. 3 Pollen tube growth and penetration on wild-type silks. **a, b** Pollen from wild-type plants. **c** Pollen from *EXPB1/expb1* plants. **d** Pollen from *expb1/expb1* plants

three silks were excised after 4 h and examined to determine the number of pollen tubes that penetrated silk hairs (Fig. 4a). Each silk contained three to five pollen grains. The results show that for the pollen that germinated, the growth and penetration into silks was not independent of the genotype of the pollen parent (Chi-square = 101;

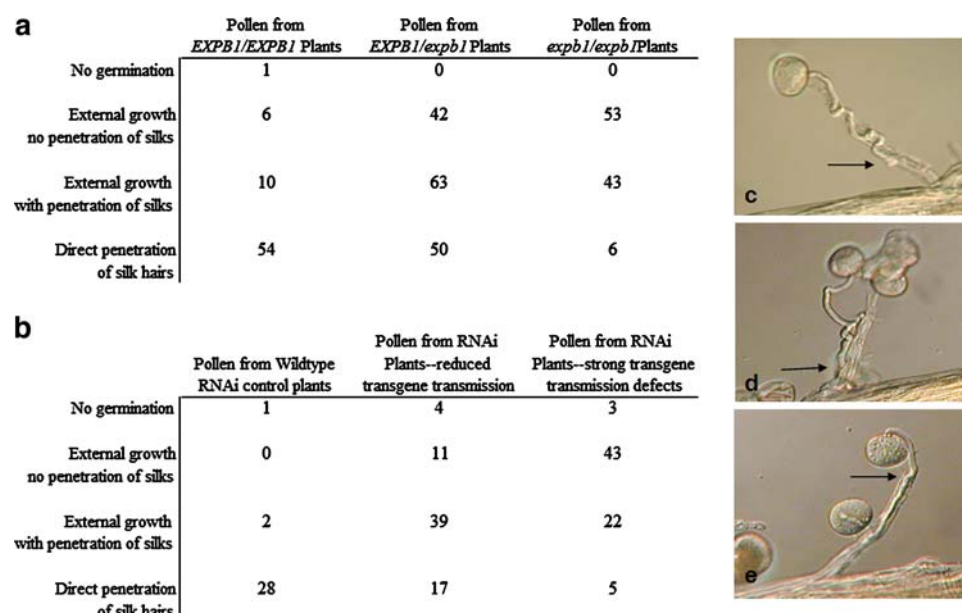


Fig. 4 Pollen penetration defects. **a** Pollen from *EXPB1/EXPB1*, *EXPB1/expb1*, and *expb1/expb1* plants on wild-type silk. **b** Pollen from wild-type RNAi control plants, RNAi plants with slightly reduced transgene transmission and RNAi plants with strongly reduced transgene transmission on wild-type silks. **c** Pollen germination and external pollen tube growth, with no penetration into the

silk hair. The *arrow* indicates the position where the pollen tube arrested growth. **d** Pollen germination and external pollen tube growth, with penetration into the silk hair at the base of the hair. The *arrow* indicates the penetration point. **e** Pollen germination and direct penetration into silk hair. The *arrow* indicates the penetration point

$df = 4$; $P \ll 0.0001$). These data indicate that *EXPB1* facilitates the initial penetration of the pollen tube into the silk, and that a delayed penetration by *expb1* pollen could be one of the main reasons for its lower reproductive success when competing against wild-type pollen.

Expression analysis of *EXPB1*

Using real-time RT-PCR, we examined the expression of *EXPB1* in pollen from *EXPB1/EXPB1* and *expb1/expb1* plants (Fig. 5). The mutant pollen showed a reduction in the expression of *EXPB1* of approximately 40% compared to the wild type. We did not expect to see a complete reduction due to the presence of three genes, *EXPB1a*, *EXPB1b* and *EXPB1c*, which are highly similar and not distinguishable by PCR. Several attempts to create gene-specific primers that would distinguish among the three genes were not successful. We conclude that *EXPB1* transcripts are significantly reduced in the mutant pollen, consistent with our previous findings that the *Mu* insertion reduced the *Zea m1d* protein by 31% (Valdivia et al. 2007b).

Phenotype of RNAi lines

In order to corroborate the linkage between the phenotype of *expb1* plants and the reduction of expansin levels, maize was transformed with an RNAi construct that was designed

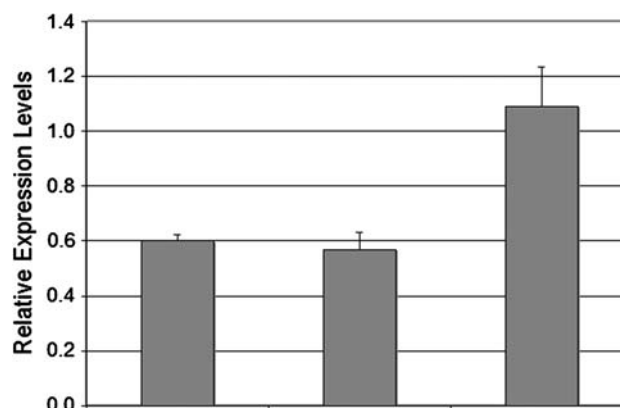


Fig. 5 Real-time RT-PCR analysis of pollen from *expb1/expb1* plants 11-6 and 11-7. Bar represents SE of three biological replicates. Primers were specific for *EXPB1a*

to silence all class B pollen β -expansin genes and was driven by a pollen-specific promoter. Twenty independently transformed calli were used to generate 70 plants. A total of 37 plants were grown to maturity at Penn State University (PSU) and 33 plants were taken to maturity at the Iowa State University Plant Transformation Facility (ISUPTF). Due to the method in which the calli were split for plantlet regeneration, each resulting plant is considered an independent transformation event. The number of transgenes present in each plant is expected to differ among plants resulting from the same callus.

When the transformed plants matured, they appeared healthy and of normal size. Reciprocal crosses with non-transformed B73 inbred (wild-type) were performed for all transformed plants, except when they did not produce pollen or silks. Plants were considered fully fertile when they produced 65 or more seeds. As much as 71% of the transgenic plants were not fully fertile when crossed as male (Table 1) and many produced reduced amounts of pollen or defective pollen. It is not unusual to find some infertile plants due to transformation artifacts; however, it is uncommon to see such a large number of infertile plants from multiple transformation events (personal communication, Tina Fonger, ISUPTF). Due to the poor quality and small amount of pollen produced by many transgenic lines, only a few of the plants grown at PSU could be analyzed. Pollen from plants grown at ISUPTF was not available. Despite these difficulties, we were able to characterize a number of lines that continued to show fertility defects as males in subsequent generations, with phenotypes similar to that found in the *Mu* insertional lines.

Defects in pollen viability and performance

In the case of line 19-1 (plants were named by the event number given by ISUPTF followed by the callus number), pollen from T₁ was viable (68% compared to 71% for wild type), but when this pollen was used to pollinate wild-type silks, no seeds were obtained. However, with the reciprocal cross (wild-type pollen on 19-1 silks), normal seed set was observed with a transgene transmission rate of 0.77 (suggesting the presence of more than one transgene insertion). The same phenotype was observed in T₂ when plants carrying the transgene were backcrossed again to wild type.

Another characteristic of pollen from line 19-1 is the formation of large aggregates, similar to those observed in the *Mu* transposon plants. Furthermore, when pollen from line 19-1 was placed on wild-type silks, pollen tubes emerged, but they did not immediately penetrate the silks (Fig. 6). The tip of the pollen tube was enlarged shortly after entering the silk, suggesting a problem with pollen tube penetration and growth through the silk.

To further examine the pollen tube penetration and growth defects, pollen from eight RNAi plants with slightly reduced transgene transmission, eight RNAi plants with strongly reduced transgene transmission, and five wild-type RNAi control plants were collected and used to pollinate wild-type silks. Two to three silks from each pollination, each silk containing three to four pollen grains, were examined and the pollen entry was categorized. The results show that for those pollen grains that germinated, the growth and penetration of silks was not

independent of the RNAi silencing (Chi-square = 102.9; $df = 4$; $P \ll 0.0001$) (Fig. 4b).

Finally, the analysis of total RNA from 19-1 pollen showed a very strong reduction in expression of class B pollen β -expansins (Fig. 7). The strong level of silencing is thus linked to a phenotype that is very similar to, but more extreme than, that seen in the *expb1* pollen (above).

In the case of line 37-5, we found very different transmission rates of the transgene through pollen and ovules in crosses from the same transgenic plant. In T₁, the transmission rate for crosses where pollen from 37-5 was used to pollinate wild-type silks was 0.29, while for the reciprocal cross the rate of transmission was 0.80. In T₂, plants from line 37-5 also had a much lower transmission rate when crossed as male (0.19), compared with the rate of transgene transmission when they were crossed as females (0.85) (Table 2). These results indicate that the presence of the transgene is detrimental to the chances that the pollen will reach the ovary and achieve fertilization, even though the level of silencing might be lower than in line 19-1.

We attempted to quantify relative expression levels of *EXPB1* in the RNAi lines by qRT-PCR, to correlate *EXPB1* expression with transgene transmission rates. However, these attempts were not successful due to lower pollen yields in the class B RNAi plants. In these plants, we also observed rapid cellular degradation that led to RNA degradation.

Defects in pollen development

In 12 plants from T₁, tassels and anthers were produced, but the anthers were shriveled and senesced before releasing pollen. The anthers from four of the 12 transformed plants (1-2, 2-3, 15-1, 15-3) and wild-type plants were examined 2–3 days before pollen shed and they showed no obvious differences, indicating that the defect appeared just before pollen shed.

In three lines from the same callus (20-1, 20-3, 20-4), the anthers also shriveled and senesced prior to anthesis. However, in these plants there were no developing pollen grains before anthesis. The same pattern was observed in the T₂ generation derived from these lines through the female. The three lines obtained from calli number 20 all produced silks and were fertile as females. Since it was not possible to obtain pollen from these plants, we were not able to characterize the pollen phenotype in more detail. This effect might be a more extreme version of the phenotype observed in the transposon insertional lines, where lack of pollen production in the greenhouse is linked with formation of pollen aggregates. The formation of pollen aggregates inside the anther might prevent pollen release, leading eventually to anthers senescing without shedding.

Table 1 Fertility of 70 independent RNAi lines for class B silencing

	No. of pollen produced	Reduced amount of pollen	Normal amount of pollen	No. of silks produced	Silks produced
No. of lines	25	22	23	16	54
No. of fertile lines	0	0	7	0	6
No. of lines w/reduced fertility	0	0	1	0	22
No. of lines w/no fertility	0	11	3	0	25
Not crossed	25	11	12	16	1

Reciprocal crosses were performed with wild type, unless transgenic pollen or silks were unavailable. Fertility was scored as seed production. Wild-type crosses performed at the same time as transgenic crosses resulted in 65–100 seed per ear. Ears that contained 65 seeds or more were considered fully fertile. Ears that contained 64 seeds or less were scored as having reduced fertility

Table 2 Transgene transmission rate from outcrosses of T1 for the class B construct

Transgenic line	Transgene transmission rate	
	Male	Female
9-2	ND	0.57 (17)
13-1	0.38 (16)	ND
17-1	NP	0.6 (15)
17-2	NP	0.26* (23)
17-3	ND	0.64 (17)
25-1	0.00* (11)	ND
25-5	0.14* (22)	0.2 (29)
25-6	NF	0.00* (10)
25-8	NF	0.00* (16)
27-1	NF	0.06* (33)
33-1	NF	0.42 (19)
33-2	0.55 (29)	0.58 (12)
33-7	NF	0.42 (24)
33-10	NF	0.36 (14)
37-5	0.19* (46)	0.85 (26)
37-6	0.4 (21)	0.7 (17)
37-10	NF	0.00* (10)
41-1	NF	0.65 (17)
41-6	0.56 (16)	0.78 (23)
41-8	0.38 (16)	0.44 (29)

Number in parenthesis indicates the number of progeny tested for the transgene

NP no pollen available, NF not fertile, ND not determined)

* Denotes rates lower than the expected 0.5 for one transgene at 95% confidence level

These results show that the effect of silencing class B pollen β -expansin genes is similar to and in some cases more extreme than the phenotype seen in the *Mu* insertion line (above). They provide additional evidence that these extracellular proteins promote pollen tube penetration and growth through the silk. Additionally, they show that reduction in β -expansin levels can prevent the correct separation of the pollen grains and block shedding, i.e., developmental roles not known from previous work.

Discussion

Our results provide a more detailed understanding of the role of group 1 allergens (pollen β -expansins) in maize pollen development and function. A common set of developmental and behavioral defects were observed in pollen with reduced gene expression of class B β -expansins, whether it was caused by a *Mu* insertion in the major *EXPB1* gene or by RNAi silencing. These defects include pollen clumping, poor pollen dispersal upon anther dehiscence and faulty penetration of the silk by the pollen tube. The difficulties that pollen bearing the *expb1* mutation have in penetrating the silk could by itself explain a large part of the delay in fertilization, and the consequent reduction in reproductive success under competitive conditions (Valdivia et al. 2007b). Reduced pollen performance was also found in three RNAi lines that showed lower transgene transmission rates through pollen than through ovules. In some cases, the reduced performance seems to affect all pollen grains. The presence of multiple independent insertions could provide a partial explanation. It is also possible that the Ory S1 promoter could have some activity before meiosis in our constructs, although in rice it seems to be active only in developing pollen grains (Xu et al. 1995, 1999). In this case, expression of the silencing construct might be predicted to induce RNA-directed DNA methylation of both alleles of *EXPB1* genes, resulting in gene silencing in every pollen grain (Jones et al. 1999, 2001; Mette et al. 2000). This point is, however, uncertain.

A related defect in pollen penetration has been described for pollen that are deficient in tapetum-synthesized xylanase (Suen and Huang 2007). Arabinoxylan is the major non-cellulosic polysaccharide in grass primary walls. Similar to our results for pollen β -expansins, a lack of xylanase delays pollen tube penetration into the silk, but does not affect germination in vitro. Pollen β -expansins have been proposed to act on arabinoxylan and it is possible that they act synergistically with xylanase in weakening the arabinoxylan–cellulose network to aid the intercellular penetration of pollen tubes.

Fig. 6 Pollen behavior and pollen tube growth. **a** Pollen from wild-type plants on wild-type silk. **b, c** Pollen from 19-1 on wild-type silks. Arrows indicate swollen pollen tube tips

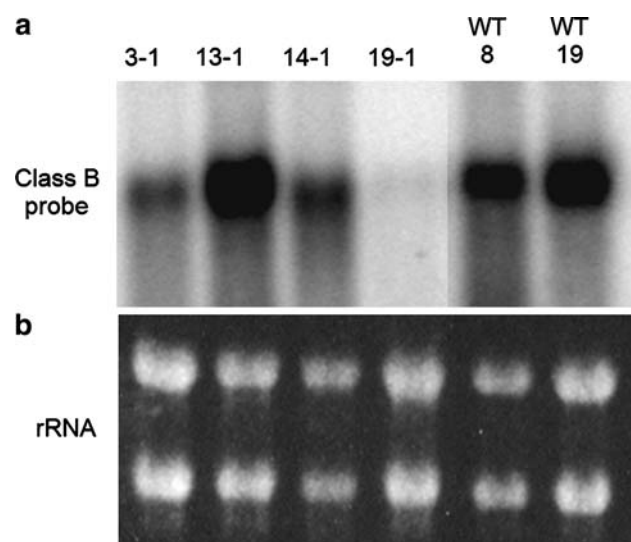
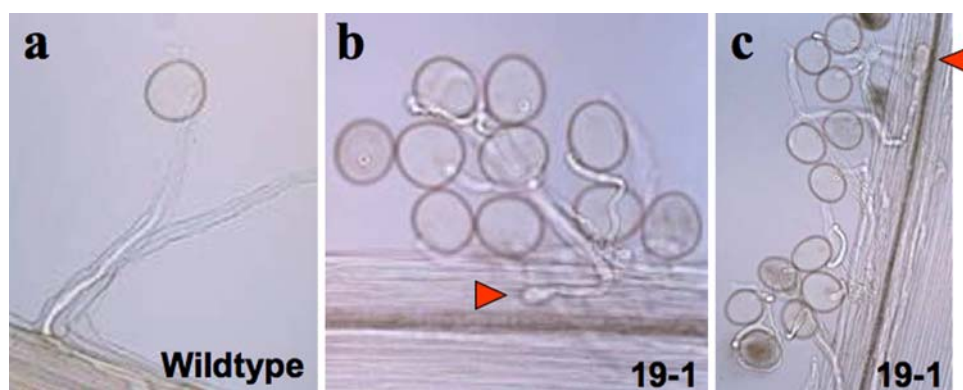


Fig. 7 Analysis of RNA from class B transgenic pollen. **a** Northern blot analysis of RNA from class B transgenic plants. **b** Agarose gel of total RNA

In both the transposon and RNAi lines, β -expansin-deficient pollen tended to form aggregates (Figs. 1, 3, 6). These observations suggest that *EXPB1* may be required for the proper separation of pollen grains just prior to anthesis. Since maize is a wind-pollinated species, the clumping of pollen could greatly reduce its ability to disperse. Heavier pollen aggregates should fall out of the air column faster than lighter pollen grains, decreasing the chances of achieving fertilization. Even if the pollen is transferred to a receptive silk and the pollen tube emerges, when stuck together, many grains are not in contact with the silk and the pollen tubes cannot reach its surface.

There are several possible explanations for the aggregation phenotype of β -expansin-deficient pollen. A recent study on the localization of *Zea m1* reported that it was found in the pollen coat and wall before germination (Wang et al. 2006). It is thus possible that a reduction of class B β -expansins causes a change in the properties of the pollen wall and coat, making it more difficult for the water

in the pollen to move out, an important step before dehiscence. This could explain the “stickiness” of the pollen walls and their tendency to form aggregates.

The transgenic lines deficient in tapetum-synthesized xylanase also showed changes in the flow of water through the pollen coat, resulting in the inhibition or delay of a key hydration step (Suen and Huang 2007). The authors suggested that the loss of xylanase on the pollen surface would impair the semifluid or amphipathic status of the coat material, which allows it to act as a waterproofing agent and also as a wick for water uptake. Similar results have been seen when lipids or certain proteins are missing from pollen (Lerouge et al. 1998). While xylanase-deficient pollen have slow hydration, pollen defective in β -expansins seem to have problems with dehydration, but the explanation could be similar. Because both xylanase and *Zea m1* are highly hydrophilic, they may influence the hydrodynamic properties of the pollen coat and wall, altering the movement of water in or out of the pollen.

A second possibility is that the reduction in class B β -expansins causes a change in the pollen developmental program through a feedback mechanism. The change in the composition or properties of the coat could potentially activate a signal transduction cascade, with the final result that pollen is unable to reach a proper dehydrated state.

Our studies have shown that class B pollen β -expansins play an important role in pollen tube penetration into the silk as well as pollen tube growth through the silk; however, we know very little about the function of the class A pollen β -expansins. The separation of class A and B occurred before the split of the maize and rice lineages (Valdivia et al. 2007a) and both classes are found in many grasses. This suggests that the two classes might be functionally specialized. It would be important to explore whether or not class A genes play a role in pollen tube penetration or perhaps they are more important for the continued growth through the transmitting tract.

The reduction of the allergen content in grass pollen could alleviate some of the health problems caused by these potent allergens and has been previously attempted

for other species (Bhalla et al. 1999). However, our data suggest that the complete elimination of these allergens may have severe detrimental effects on the reproductive success of the affected species, which could render this strategy impractical in many cases. In addition to slower pollen penetration and growth, the reduction in pollen allergens may affect pollen maturation through changes in the properties of wall and coat that can lead to pollen aggregation or even failed dehiscence.

Acknowledgments We thank: T. Omeis for the use of the Buckhout Greenhouse; R. Oberheim and the Department of Horticulture for use of the Agricultural Experimental Station at Rock Springs, PA; Dr. R. Meeley from Pioneer Hi-Bred International, Inc. for the original transposon insertion lines; Dr. K. Wang of the Plant Transformation Facility at Iowa State University for assistance in maize transformation; Dr. P. Bhalla for the *ory* *SI* promoter; Drs S. Chopra, D. Braun and P. McSteen for field space, helpful discussions and guidance; Dr. D. Grove for design of the primers and probes and for running the RT-PCR assays at the Nucleic Acid Facility (Huck Institute, Pennsylvania State University); and J. Sampedro, E. Wagner, T. Kinney, N. Sella Kapu and J. Mena-Ali for assistance in the field, greenhouse and laboratory. This project was made possible, in part, through the use of technology developed by Pioneer Hi-Bred International, Inc. This research was supported by NIH grant GM060397 and DOE grant DE-FG02-84ER13179 to D. J. C.; by NSF grant DEB 02-35217 and USDA grant 2005-35320-15251 to A. G. S.; and E. R. V. was supported in part by an NSF Graduate Research Training grant.

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Supplemental Table 1.

Supplementary Table 1. Primers and probes that were used in real-time PCR analysis. F= forward primer; R= reverse primer; *= labeled at 5' end with 6-FAM and at 3' end with BQH-1.

Primer	Sequence (5'-3')	Annealing Temperature °C
EXPB1-F	AATTCCCTTCGATTCATCCG	58
EXPB1-R	GCTGCCAACTTAATTAGCTTGC	
EXPB1 probe*	CACAGCCAACTTAATTAGCTTGC	
GADPH-F	TGACAGCAGGTCGAGCATCT	58
GADPH-R	TTGACGAAGTGGTCGTTTCAGAG	
GADPH probe*	CGACGCCAAGGCCGGGATC	