A Group-1 Grass Pollen Allergen Influences the Outcome of Pollen Competition in Maize

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Worldwide, 400 million people suffer from hay fever and seasonal asthma. The major causative agents of these allergies are pollen specific proteins called the group-1 grass pollen allergens. Although details of their antigenicity have been studied for 40 years with an eye towards immunotherapy, their function in the plant has drawn scant attention. Zea m 1 constitutes a class of abundant grass pollen allergens coded for by several genes that loosen the walls of grass cells, including the maize stigma and style. We have examined the impact of a transposon insertion into one of these genes (EXPB1, the most abundant isoform of Zea m 1) on the production of Zea m 1 protein, pollen viability, and pollen tube growth, both in vitro and in vivo. We also examined the effect of the insertion mutation on the competitive ability of the pollen by experimentally varying the sizes of the pollen load deposited onto stigmas using pollen from heterozygous plants and then screening the progeny for the presence of the transposon using PCR. We found that the insertional mutation reduced the levels of Zea m 1 in maize pollen, but had no effect on pollen viability, in vitro pollen tube growth or the proportion of progeny sired when small pollen loads are deposited onto stigmas. However, when large pollen loads are deposited onto the stigmas, the transposon mutation is vastly underrepresented in the progeny, indicating that this major pollen allergen has a large effect on pollen tube growth rates in vivo, and plays an important role in determining the outcome of the pollen-pollen competition for access to the ovules. We propose that the extraordinary abundance (4% of the extractable protein in maize pollen) of this major pollen allergen is the result of selection for a trait that functions primarily in providing differential access to ovules.

INTRODUCTION

Each silk (stigma/style) of a maize plant can support the germination and growth of numerous pollen tubes, but only one tube enters the micropyle, penetrates the ovule and achieves fertilization. Considering that only the first pollen tube to reach the micropyle passes its genes to the next generation, it is not surprising that the entire process is very fast. In maize, rehydration and germination of the pollen grain occur within 5 min of deposition on the silk, and pollen tubes grow at rates exceeding 1 cm h⁻¹ [1]. Even the longest silks that are connected to the lowermost ovaries on an ear are traversed in 24–30 h. To make this trek (up to 40 cm in maize), the male gametophyte must transcribe and translate a large number of genes. In mature maize pollen, an estimated 24,000 genes are expressed by the microgametophyte, of which 10% are pollen-specific [2]. Recent studies of transcript profiling in pollen indicate even higher percentages of pollen-specific gene expression [3], although the vast majority of genes expressed by microgametophytes still appear to be expressed during both the sporophytic and gametophytic stages of the life cycle. Because genes that give a competitive advantage in the race from the stigma to the ovule are expected to increase in the population, it is reasonable to predict that at least some of the pollen-specific genes have evolved in response to pollen-pollen competition for access to the ovules.

The group-1 grass pollen allergens are pollen-specific proteins originally identified by immunologists 40 years ago as the main causative agents of hay fever and seasonal asthma induced by grass pollen [4–6]. Although many details of their antigenicity have been studied with an eye towards immunotherapy [7–10], their function in the plant has drawn scant attention. Recently these pollen allergens were recognized as members of a subclass of the β-expansin family [11,12]. Expansins comprise a large superfamily of proteins that characteristically loosen the plant cell wall by weakening the noncovalent bonding of polysaccharides to one another [13–16]. The individual members of this superfamily are known to play important roles in plant growth and development [14,17,18]. The group-1 allergens from maize pollen, collectively known as Zea m 1, are highly abundant glycoproteins, constituting ~4% of the protein extracted from pollen; they are rapidly secreted upon pollen hydration and have wall-loosening activity specific for grass cell walls [12,19]. Because Zea m 1 (and its homologs in other grass species) and the mRNA from the genes that encode it have only been found in grass pollen, it is thought to exhibit pollen-specific gene expression [20,21]. In this study, we assessed the role of Zea m 1 in pollen viability, pollen tube growth, and pollen competitive ability by use of a maize line containing

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a *Mu* transposon insertion in *EXPB1* (GenBank Accession AY197353), a gene that codes for Zea m 1d, the most abundantly expressed of four Zea m 1 isoforms [19]. We found that the reduction of Zea m 1 caused by the insertion has a strong effect on pollen tube growth in vivo and the ability of pollen to achieve fertilization under conditions of pollen competition.

**RESULTS**

**Effects of the *Mu* Insertion on Zea m 1 Production in Pollen**

From a large library of maize lines bearing Robertson’s Mutator (*Mu*) insertions (obtained from Pioneer Hi-Bred International, Inc., Johnson, IA, USA; see [22–24]), we identified a single line with a *Mu* insertion in *EXPB1* (Figure 1). From this line we created, via repeated backcrosses into the non-mutator parental line 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. Analysis of pollen protein extracts by two-dimensional gel electrophoresis and immunoblotting from *EXPB1/EXPB1* and *expb1/expb1* plants revealed that overall Zea m 1 production was reduced by 31% in *expb1* pollen compared with the overall production of Zea m 1 in *EXPB1* pollen (Figure 1c,d). It should be noted that we did not expect Zea m 1 production or even the Zea m 1d isoform, to be completely eliminated in the pollen from *expb1/expb1* plants because multiple genes contribute to the Zea m 1 pool and recent evidence indicates that additional genes highly similar to *EXPB1* (>98.8% nucleotide sequence identity) also code for the same isoform [19,21].

**Effects of the *Mu* Insertion on Pollen Viability and Pollen Performance**

Thiazolyl blue staining of pollen revealed that the reduction in the overall pool of Zea m 1 in *expb1* pollen does not seem to affect the viability of the pollen produced by *expb1/expb1* plants. An analysis of variance (ANOVA) revealed no significant effect of plant genotype on pollen viability ($F_{2,59} = 0.92; p = 0.4$). For each of the three maize genotypes (*EXPB1/EXPB1, EXPB1/expb1, expb1/expb1*) $75\text{-}78\%$ of the pollen stained a deep purple (Figure 2a,b).

When pollen from 20–22 plants from each of the 3 genotypes were germinated and grown on a medium in Petri plates [25], there were significant effects of plant genotype on the mean per plate in vitro growth of pollen (ANOVA, $F_{2,59} = 4.97; p = 0.01$). Tukey pairwise comparisons with adjusted probabilities for multiple comparisons revealed that there was no significant difference in the in vitro growth of pollen tubes from *expb1/expb1* and *EXPB1/EXPB1* plants but that the pollen from the *EXPB1/expb1* plants grew faster in vitro (Figure 2c) most likely a general result of heterosis, reflecting the hybrid's greater vigor and ability to provision the pollen grains during development [see [26–28] for a discussion of the effects of nutrient and energy storage com-

![Figure 1. Mu insertion into EXPB1 and its effect on Zea m 1 content of pollen. (a) Cartoon showing the structure of EXPB1 and location of the Mu insertion (exons denoted with boxes). Also indicated are the locations of primers used for PCR screening. (b) Mu is inserted near the intron border flanking the fourth exon. (c) Portion of a 2-D gel image of wild type (EXPB1) pollen protein showing the Zea m 1 isoforms, which were identified by immunoblotting. (d) Relative amount of total Zea m 1 protein extracted from pollen of EXPB1/EXPB1 and expb1/expb1 plants. (Mean±SE; N = 2; t = 9.15; p = 0.035). doi:10.1371/journal.pone.0000154.g001](figures/1.png)
pounds provided by the paternal sporophyte during development on initial pollen tube growth rates and for examples of other species which exhibit similar (i.e., heterosis/inbreeding) effects on pollen tube growth). The lack of a significant difference between the two true breeding lines, however, indicates that in vitro pollen tube growth is not affected by the Mu insertion. Moreover, there is no hint of a bimodal distribution in the in vitro growth of the pollen tubes from the heterozygous plants as would be expected if EXPB1 and expb1 pollen grew at different rates in vitro (data not shown).

To determine the ability of EXPB1 and expb1 pollen to achieve fertilization under conditions of pollen competition, we varied the volume (number) of pollen grains from EXPB1/expb1 plants deposited onto the silks of EXPB1/EXPB1 plants. We found that the transmission of expb1 depended upon the volume of pollen grains used in the pollination ($\chi^2 = 53.2$; df = 3; p<0.0001) (Figure 3). When the 50 and 100 $\mu$L volumes were used in the pollination, fertilization was random (nearly 1:1) with respect to the EXPB1 allele carried by the pollen. In contrast, when the two largest volumes of pollen were used in the pollination, the expb1 gene was significantly underrepresented in the progeny (only 3% of the seeds following pollination with the largest volume), indicating that as the intensity of pollen competition increases, the proportion of seeds sired by expb1-bearing pollen decreases.

Experiments designed to directly examine in vivo pollen tube growth rates indicate that these differences in the ability to achieve fertilization under competitive conditions are due to differences in pollen tube growth rates. When we examined the silks at 8 h after pollination, we found that silks pollinated with EXPB1 pollen contained a significantly greater number of pollen tubes (2.18±0.09; mean±SE) at 8 cm below the site of pollen deposition than silks pollinated with expb1 pollen (0.98±0.06) (ANOVA, F$_{1,7}$ = 120.9; p<0.0001). At 22 h after pollination, we found pollen tubes in 37.5% of the ovaries following pollinations by EXPB1 pollen (Figure 4). In contrast, we found no pollen tubes in the ovaries at 22 h after pollination when the silks were pollinated by expb1 pollen. Together these data indicate that the expb1 pollen grows more slowly in vivo than the EXPB1 pollen.

**DISCUSSION**

Competition among males for access to the ova/ovules of females is thought to have shaped the haploid phase of the life cycle in both animals (the ejaculate and sperm) and higher plants (the male gametophyte/pollen) (see [29] for recent review). In plants, the pollen load that accumulates on a stigma frequently consists of the pollen from several individuals and often exceeds the number of grains necessary to fertilize all of the ovules [30–34]. Consequently, the pollen from various individuals is placed into a competitive circumstance of great evolutionary importance: only those pollen grains that germinate and grow the fastest through the maternal tissue of the stigma, style and ovary will penetrate the ovule and fertilize the egg. Our data indicate that EXPB1 plays a large role in generating the rapid in vivo growth rates of maize pollen tubes.

Figure 2. Pollen viability and pollen performance in vitro and in vivo. (a) Percentage of viable pollen, based on staining with thiazolyl blue (mean±SE, N = 20–22 plants). (b) Micrograph of pollen stained with thiazolyl blue. Viable pollen stained dark purple. (c) Pollen tube growth in vitro (mean±SE, N = 20–22). Bars with different letters of the alphabet differ significantly using Tukey pairwise comparisons with the overall probability adjusted for multiple comparisons.

doi:10.1371/journal.pone.0000154.g002
Our findings reveal that a 31% reduction in Zea m 1 caused by a transposon insertion into the EXPB1 gene (the most abundant isoform of Zea m 1) has no significant effects on pollen viability or in vitro pollen tube growth rates but has a large effect on in vivo pollen tube growth rates and the ability to achieve fertilization under conditions of pollen competition. Because the pollen competition experiment varied the size of the pollen load produced by heterozygous (EXPB1/expb1) plants rather than using the pollen produced by the true breeding (homozygous) plants, the results are not an artifact of differences in vigor between the true breeding lines. This experimental design, however, does not exclude the possibility that loci distinct from EXPB1 could account for our results. After backcrossing the original line with the Mu insertion to the non-mutator parental line for 3 generations, the resulting plants still contain, on average, 12.5% of the genes from the original Mu insertion line. The vast majority of these genes would randomly segregate into the EXPB1 and expb1 pollen produced by the heterozygous plants used in the pollen competition experiment. Only those genes that consistently cosegregate with expb1 could potentially influence performance of expb1 pollen. Because only 3% of the seeds produced by our largest pollen load contained expb1, another mutation that is responsible for the large effect on pollen performance that we observed would have to lie no more than 3 cM away from the EXPB1 locus to cause this skewed ratio. The EXPB1 locus is located on chromosome 9 which has a genetic distance of approx. 150 cM [35]. Consequently the region that cosegregates with the EXPB1 locus represents <4% of the genetic distance of chromosome 9 in maize (chromosome n = 10). It would be extremely unlikely to find in this small region a second mutation with the precise phenotype described in this paper. This caveat aside, our findings suggest that the β-expansin encoded by EXPB1 does not perform a vital role in pollen development or in the internal growth processes of the pollen tube per se. Previous in vitro studies demonstrated that Zea m 1 loosens the cell walls of silks and other studies showed that Zea m 1 is secreted by pollen upon hydration and tube growth [12,19]. These findings support the inference that Zea m 1 assists pollen tube penetration by loosening the maternal cell walls of stigma/style. Our data also provide strong, additional evidence that EXPB1 is gametophytically expressed because the performance of pollen from heterozygous plants depended upon whether the pollen carried the EXPB1 or the expb1 allele. If the protein (or the mRNA) from EXPB1 was sporophytically produced (e.g., by the tapetum) and then moved into the pollen, there would be no difference in the performance of pollen bearing different alleles. A few pollen expressed genes have already been shown to affect pollen tube growth rates in vivo (see reviews by [36,37]) including genes that are expressed during both stages of the life cycle (e.g., [38,39]) and a few pollen specific genes that play important roles in the internal growth processes of the pollen tube [40,41]. In addition, several studies have shown for genes expressed during both stages of the life cycle that selection on the microgametophyte (e.g., for cold tolerance or herbicide resistance) can alter the proportion of progeny with the selected trait (e.g., [32,42–46]).

Although expansins comprise a large family of genes whose proteins play diverse roles in plant growth and development in vitro.
In order to assess the impact of the Mu insertion into the EXPB1 gene on Z. m. 1 production, maize pollen was collected in July 2004 from EXPB1/EXPB1 and expb1/expb1 plants grown at The Pennsylvania State University Agricultural Experiment Station at Rock Springs PA (near State College, PA), cleaned by passing through a series of sieves, and stored separately at −80°C. Approximately 25 mg of maize pollen was extracted in 4 volumes (0.1 mL) of 50 mM sodium acetate, pH 4.5, for 1 h at 4°C. The extract was centrifuged at 20,000 g for 10 min. Proteins in the supernatant were quantified colorimetrically with the Coomassie Plus® Protein Assay Reagent (Pierce, Rockford, IL) according to the manufacturer’s instructions.

These proteins were then subjected to 2-dimensional gel electrophoresis. For the first dimension – isoelectric focusing (IEF) – Immobiline DryStrip gels (pH 6–11, 11 cm) and IPG buffer (pH 6–11) were obtained from GE Healthcare Bio-Sciences Corp. (Piscataway, NJ). The gels were rehydrated for 16 h with the rehydration buffer (8 M urea, 2% CHAPS, 0.5% IPG buffer, 0.002% bromophenol blue) containing the protein extracts and then focused in a PROTEAN IEF cell apparatus (Bio-Rad Laboratories, Hercules, CA) at the following program: running temperature: 20°C; maximum current: 50 μA/gel; Step 1: 200 V for 30 min (linear ramp); Step 2: 300 V for 30 min (linear ramp); Step 3: 8,000 V for 150 min (linear ramp); Step 4: 8,000 V for 55,000 Vh (linear ramp). After the completion of IEF, the gels were incubated for 15 min in SDS equilibration buffer (50 mM Tris-HCl, pH 8.8, 6 M urea, 30% glycerol, 2% SDS, and 0.002% bromophenol blue) with 10 mg/mL dithiothreitol and then switched into the same buffer containing 25 mg/mL iodoaceta-mide for another 15 min. For the second dimension, proteins were separated by discontinuous SDS-PAGE in a Criterion Dodeca Cell apparatus (Bio-Rad Laboratories, Hercules, CA) using 12.5% precast gels. 2-D gels were stained with protein with SYPRO Ruby (Bio-Rad Laboratories, Hercules, CA) according to the manufacturer’s instructions and quantified using a laser scanner (Molecular Imager FX Pro PLUS from Bio-Rad) and 2-D image analysis software (PDQuest Version 7.3 from Bio-Rad). The protein marker, Mark12 Unstained Standard, for SDS-PAGE, was from Invitrogen Inc. (Carlsbad, CA; Catalog No. LC5677).

To identify the β-expansins, the resulting 2-D gels were then subjected to immunoblot analysis. This analysis was performed in a Bio-Rad Criterion blotter as described by Li et al. [19]. For immunodetection of Z. m. 1, both monoclonal and polyclonal antibodies against Lol p 1 were used. Lol p 1 is the group-1 allergen of perennial ryegrass pollen and has a sequence similarity to Z. m. 1. The SeeBlue Plus2 Pre-Stained Standard was purchased from Invitrogen (Carlsbad, CA; Catalog No. LC5925).

**PolLEN Viability, and Pollen Tube Growth In Vitro and In Vivo**

To assess the impact of the Mu insertion into EXPB1 on pollen viability, we collected pollen at anthesis from 20–22 plants from each of the three genotypes (EXPB1/EXPB1 (N = 20), EXPB1/expb1 (N = 22), expb1/expb1 (N = 20) plants), stained it with thiazolyl blue to assess membrane integrity—a trait that is highly correlated to germinability [32], and counted the number of stained pollen grains in a sample of 100 grains per plant. Pollen of the appropriate genotype was placed onto a slide, stained under a cover slip and observed under a microscope at 30×. The first 100 grains were scored in a left to right transect starting in the left center of the cover slip. We also assessed the in vitro growth of pollen tubes from each of the three genotypes by sprinkling the pollen from 20–22 plants of each genotype onto Petri plates containing a maize pollen germination and pollen tube growth media [23]. After 20 minutes at 28°C a few drops of 80% ethanol was added to each plate to stop pollen tube growth; the Petri plates
were placed under a dissecting scope at 8×; and the lengths of the first 20 pollen tubes encountered in the left transect starting in the left center of the plate were recorded using image analysis [59].

To determine the effect of the Mu insertion into EXPB1 on the competitive ability of pollen in vivo, we experimentally manipulated the intensity of competition between mutant and wild type pollen. Pollen from field grown, heterozygous (EXPB1/EXPb1) plants was collected, cleaned, aliquoted into 50, 100, 250 and 500 μL volumes, and sprinkled over virgin silks of true breeding wild type plants (four replicate pollinations per volume, yielding 16 ears). We reasoned that under conditions of intense pollen competition (e.g., the 500 μL sample of pollen) only the fastest growing pollen tubes would achieve fertilization, whereas under conditions of little or no pollen competition (e.g., the 50 μL sample) both the fast and slowly growing pollen would achieve fertilization. A random sample of 30 seeds from each of the 16 ears (480 progeny total) was assessed for the presence of the mutant allele (EXPb1) by PCR.

We also directly examined the growth of EXPB1 and EXPb1 pollen tubes through maize silks. We pollinated the silks of 16 wild type plants (EXPB1/EXPB1) with pollen from either EXPB1/EXPB1 or EXPb1/EXPb1 plants. On eight plants (four from each type of pollination), we removed the silks after 8 h; stained the silks with 0.1% aniline blue for 30 min and examined 10–14 silks from the region from 7.5 to 8.5 cm from the site of pollen deposition using fluorescence microscopy [54]. On the remaining eight plants we examined 10 ovaries from the central region of the ear for the presence of a pollen tube at 22 h after pollination by excising the ovary, staining it with aniline blue, and examining it under a fluoresce microscopy [54].

ACKNOWLEDGMENTS

We thank T. Omeis for use of the Buckhout Greenhouse, R. Oberheim and the Department of Horticulture for use of the Agricultural Experiment Station at Rock Springs, PA, S. Chopra for field space, helpful discussion and guidance, D.M. Durachko, E. Wagner, M. Perich, J. Sampredo, R. Carey, T. Kinney, N. Sella Kapu and J. Menu-Ali for assistance in the field, greenhouse, and lab. This project was made possible, in part, through use of technology developed by Pioneer Hi-Bred International, Inc. with help from Dr. Robert Meley.

Author Contributions

Conceived and designed the experiments: AS EV LL DC. Performed the experiments: EV YW LL. Analyzed the data: AS EV. Contributed reagents/materials/analysis tools: DC. Wrote the paper: AS DC. Other: Assisted in manuscript preparation: L-CL DC EV.

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