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EXPORTIN1 Genes Are Essential for Development and Function of the Gametophytes in *Arabidopsis thaliana*

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ABSTRACT

Gametes are produced in plants through mitotic divisions in the haploid gametophytes. We investigated the role of *EXPORTIN1* (*XPO1*) genes during the development of both female and male gametophytes of Arabidopsis. Exportins exclude target proteins from the nucleus and are also part of a complex recruited at the kinetochores during mitosis. Here we show that double mutants in Arabidopsis *XPO1A* and *XPO1B* are gametophytic defective. In homozygous–heterozygous plants, 50% of the ovules were arrested at different stages according to the parental genotype. Double-mutant female gametophytes of *xpo1a-3/+*; *xpo1b-1/xpo1b-1* plants failed to undergo all the mitotic divisions or failed to complete embryo sac maturation. Double-mutant female gametophytes of *xpo1a-3/xpo1a-3*; *xpo1b-1/+* plants had normal mitotic divisions and fertilization occurred; in most of these embryo sacs the endosperm started to divide but an embryo failed to develop. Distortions in male transmission correlated with the occurrence of smaller pollen grains, poor pollen germination, and shorter pollen tubes. Our results show that mitotic divisions are possible without XPO1 during the haploid phase, but that XPO1 is crucial for the maternal-to-embryonic transition.

THE life cycle of flowering plants alternates a diploid sporophytic phase with a highly reduced haploid phase. Within the reproductive structures of the flower, specialized cells undergo meiosis and subsequent mitoses to form the female and the male gametophytes named, respectively, the embryo sac and the pollen grain. A typical embryo sac consists of seven cells: three antipodal cells, a diploid central cell, an egg cell, and two synergid cells (reviewed in Drews and Yadegari 2002). The pollen grain is composed of a vegetative cell and a generative cell, which divides to produce two sperm cells. Upon hydration on the stigma, the vegetative cell produces a tube that grows by tip extension to transport the sperm cells into the embryo sac. In the embryo sac, the synergids, in particular, play an important role in attracting the pollen tube and in the discharge of the sperm cells (Higashiyama *et al.* 2003; Huck *et al.* 2003; Rotman *et al.* 2005). At fertilization, one sperm cell fuses with the central cell to produce the endosperm and the other sperm cell fuses with the egg cell to produce the zygote (reviewed in Boavida *et al.* 2005).

In eukaryotes, the bilayered nuclear membrane represents a selective barrier separating the nucleoplasm from the cytoplasm. Directional transport of macromolecules across that membrane is mediated by karyopherins, which interact with their cargo through two types of signaling sequences: nuclear localization signals that lead to nuclear entry and nuclear export signals (NES) that regulate exit from the nucleus (reviewed in Meier 2005). Chromosome region maintenance 1/exportin 1 (CRM1/XPO1) was originally identified in the fission yeast *Schizosaccharomyces pombe* in a genetic screen for cold-sensitive mutants affected in their chromosomal structure (Adachi and Yanagida 1989). CRM1/XPO1 is involved in two cellular processes: the control of mitosis and the nuclear export of target proteins and messenger RNAs. During mitosis, CRM1/XPO1 acts with Ras-related nuclear–guanosine triphosphate (Ran–GTP) and recruits partner proteins in complexes connected with the kinetochore at centromeres (Arnaoutov *et al.* 2005; Wang *et al.* 2005). The lack of CRM1 was associated with centromere reduplication and defects in the segregation of the chromosomes, thus precluding cellular divisions. CRM1/XPO1 is also the main nuclear export receptor, recognizing a broad range of NES-bearing substrates (Fornerod *et al.* 1997; Kudo *et al.* 1997; Stade *et al.* 1997). Nuclear exclusion of substrates can be abolished by various factors that affect the affinity of XPO1 for a specific cargo (reviewed in Merkle 2003).

In recent years, we have been studying several nucleocytoplasmic proteins that enhance tolerance to metal and oxidative stresses. These factors relocate to the nucleus in the presence of leptomycin B, a specific

Sequence data from this article have been deposited with the EMBL/GenBank Data Libraries under accession nos. At5g17020 (*XPO1A*), At3g03110 (*XPO1B*), SALK_028886 (*xpo1a-1*), SALK_086909 (*xpo1a-2*), SALK_078639 (*xpo1a-3*), and SALK_088267 (*xpo1b-1*).

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inhibitor of XPO1 (Yoshida and Horinouchi 1999). To verify the dependence of these proteins on XPO1-mediated nuclear export, we attempted to recover a mutant lacking XPO1. In Arabidopsis, *XPO1* was identified as a single gene (Haasen *et al.* 1999), but a second locus was later identified in the genome (Arabidopsis Genome Initiative 2000). The two loci were renamed *XPO1A* (At5g17020) and *XPO1B* (At3g03110) and the corresponding proteins share 86% identity (supplemental Figure S1). In a reverse genetics approach, single mutants appeared normal, so we concluded that each paralogous gene could functionally mask the loss of the other. However, a double-mutant homozygote was not recovered. Distortions from expected segregation ratios indicated that the double-mutant gametes were counterselected. Cotransmission of mutant alleles was abolished through the female and strongly reduced through the male. Female gametophytes that were mutant at both loci showed defects ranging from early developmental arrests to disorganized cellular constitutions. Depending on the genotype of the maternal sporophyte, zygotes could be produced, but they did not develop into embryos. We therefore concluded that a maternal copy of *XPO1* is required for establishing a viable embryo.

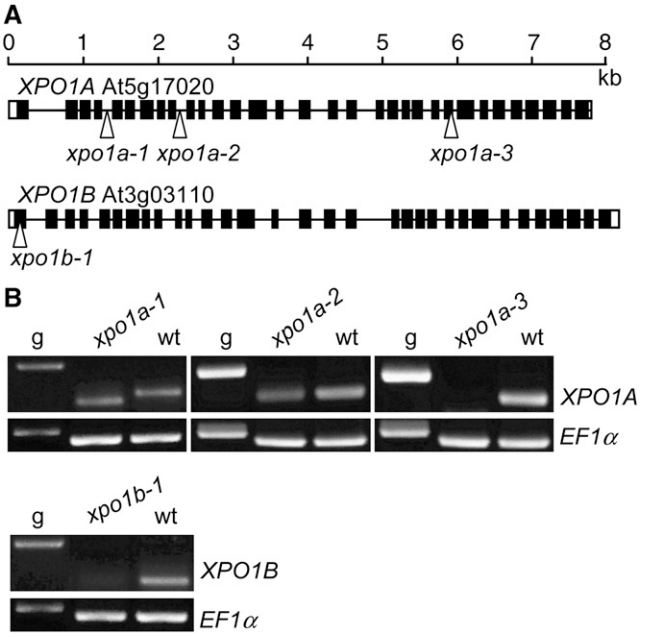

Figure 1.—Structure of *XPO1* genes, mutant alleles, and expression analysis. (A) Solid boxes, exons; triangles, T-DNA insertions in *xpo1a-1* (intron 4, 1304 bp from the transcription initiation start), in *xpo1a-2* (intron 9, 2417 bp from the transcription initiation start), in *xpo1a-3* (exon 24, 5919 bp from the transcription initiation start), and in *xpo1b-1* (exon 1, 74 bp from the transcription initiation start). (B) RT–PCR analysis of *xpo1a-1*, *xpo1a-2*, *xpo1a-3*, *xpo1b-1*, and wt (wild type) seedling RNA. g, genomic DNA control.

MATERIALS AND METHODS

Plant materials and genotyping: Arabidopsis seeds of *xpo1a* and *xpo1b* alleles (*xpo1a-1*, SALK_028886; *xpo1a-2*, SALK_086909; *xpo1a-3*, SALK_078639; *xpo1b-1*, SALK_088267) from T-DNA mutagenesis populations (Alonso *et al.* 2003) were obtained from the Arabidopsis Biological Resource Center and the position of the T-DNA insertions was confirmed by PCR and sequencing. The presence of the T-DNA (T) and the wild-type allele (G) was tested by PCR using the primers *xpo1a-1* [T (oXa1R, oLBa1) G (oXa1F, oXaR)]; *xpo1a-2* [T (oXa2R, oLBa1) G (oXa2F, oXa2R)]; *xpo1a-3* [T (oXa3F, oLBa1) G (oXa3F, oXa3R)]; and *xpo1b-1* [T (oXb1F, oLBa1) G (oXb1F, oXb1R)] (supplemental Table S1).

Reciprocal crosses and genetic analysis: The transmission efficiency (TE) through the female or male gametophytes was determined using reciprocal backcrosses. TE was defined as the number of mutant alleles divided by the number of wild-type alleles in the progeny plants. Stage 12 flowers (Smyth *et al.* 1990) were emasculated and pollinated 24 hr later. The genotype of individual progeny plants was determined by PCR analysis.

Phenotypic analysis: Pollen morphology was analyzed by spreading pollen from one or two flowers onto a strip of double-sided tape attached to a slide. Histochemical tests were performed on mature pollen from five to eight flowers that were collected in liquid pollen germination medium as described (Becker *et al.* 2003). Alexander's stain for pollen viability was according to Johnson-Brousseau and McCormick (2004). Incubation in 1 μg/ml 4′,6-diamidino-2-phenylindole (DAPI) was used to determine the cellular constitution of the pollen. Pollen germination assays were performed on solid medium as described (Boavida and McCormick 2007). Assays were repeated at least three times. To determine if pollen tubes were correctly attracted to the ovule, pistils were dissected with a needle to expose ovules; the pistils were fixed 20 min in FAA (3.5% formaldehyde, 5% acetic acid, 50% ethanol), incubated 16 hr in 8 m NaOH, washed twice in water, and stained 16 hr with decolorized aniline blue (DAB). Seed set and the percentage of abnormal seeds were determined using at least five siliques. For ovule clearing, 2- to 4-day-old siliques were dissected by cutting on both sides of the replum using a 30-gauge syringe needle. Ovules were fixed 20 min in FAA, washed 5 min in water, and cleared 16 hr in Hoyer's solution (7.5 g gum arabic, 100 g chloral hydrate, and 5 ml glycerol in 30 ml water) (Meinke 1994). Images were acquired using a Zeiss Axiophot microscope with differential interference contrast optics (DIC); images were captured with Axiovision 4.3 software using an Axiocam MR camera.

Cloning of *XPO1A* ORF: cDNA was synthesized from RNA prepared from seedlings of wild-type Arabidopsis L*er*. The *XPO1A* open reading frame was PCR amplified in four different fragments—(α: oX1kpnI, oX3), (β: oX2, oX5), (γ: oX4, oX7), and (δ: oX6, oX8bamhI)—using Phuzion (Finnzymes) and cloned into pGemT (Promega). The fragments were then combined using internal enzyme sites within the *XPO1A* cDNA sequence in three steps as (α *Sph*I β), (γ *Spe*I δ), and then (αβ *Apa*I γδ) to obtain the full-length ORF. The *Kpn*I–*Bam*HI fragment was then transferred in pEZS-NL (D. Ehrhardt, Carnegie Institution, Stanford, CA) for fusion with GFP. The 35S:XPO1::GFP expression cassette was then cloned in the binary vector pART27 (Gleave 1992). The binary vector was transformed into *Agrobacterium tumefaciens* strain GV3101 for floral dip infiltration of *Arabidopsis thaliana* (Bechtold *et al.* 1993).

RT–PCR analysis and PCR-based transmission analysis: Unpollinated pistils were harvested 1 day after emasculation and pollen grains were vacuum harvested (Johnson-Brousseau and McCormick 2004). RNA extraction and reverse transcrip-

TABLE 1

Transmission efficiency of *xpo1* alleles in single mutants

Female × male	F_2 genotypes: *a/a; b1/b1*	F_2 genotypes: *a/+; b1/b1* *a/a; b1/+*	F_2 genotypes: Other	HoHz[a] (%)
a1/a1 × *b1/b1*	0	3	69	4.2
a2/a2 × *b1/b1*	0	21	63	25.0
a3/a3 × *b1/b1*	0	3	218	1.4

[a] HoHz are plants *ho*mozygous at one locus and *he*terozygous at the other locus. While both HoHz's were obtained in the F_2 from the *a3/a3* × *b1/b1* cross, only *a1/+ b1/b1* was obtained in the screened population, as a consequence of the observed very low cotransmission efficiency.

tion was conducted using an RNeasy plant mini kit from Qiagen and reverse transcriptase Superscript III from Stratagene. PCR was conducted using specific primers for *XPO1A* (oXa3F, oXa3R), *XPO1b* (oXb1F, oXb1R), and *AtACT2* (act2.3, act2.5). Ovules were dissected from pistils 1–3 days after the backcross and single ovules were directly immersed in the PCR reaction mix.

RESULTS

Identification and characterization of mutant lines: To assess the role of the *XPO1* genes in *A. thaliana*, three *XPO1A* alleles (*xpo1a-1*, *xpo1a-2*, and *xpo1a-3*) and one *XPO1B* allele (*xpo1b-1*) (Figure 1) were obtained from T-DNA collections. None of the single-mutant homozygous plants showed any obvious alterations or notable transmission defects. Expression analysis in the homozygous plants was conducted by RT–PCR, using primers across each insertion. In *xpo1a-3* (*a3*) or *xpo1b-1* (*b1*), a transcript was not detected, indicating that they represent null alleles. In *xpo1a-1* (*a1*), a shorter transcript was found, which we deduced to have resulted from aberrant splicing that led to a 72-bp in-frame deletion (loss of amino acids 104–127). In *xpo1a-2* (*a2*), the T-DNA insertion into the intron did not change the size of the transcript.

TABLE 2

Transmission efficiency of *xpo1* alleles and reciprocal crosses

Parental genotype	F_1 genotypes (self): *a/a; b1/b1*	*a/+; b1/b1*	*+/+; b1/b1*	% mutant allele[a]
a1/+; b1/b1	0	5	67	(a1) 3.4
a2/+; b1/b1	16	29	27	(a2) 42.4
a3/+; b1/b1	0	28	243	(a3) 5.2
Parental genotype	F_1 genotypes (self): *a3/a3; b1/b1*	*a3/a3; b1/+*	*a3/a3; +/+*	% mutant allele[a]
a3/a3; b1/+	0	26	179	(b1) 6.3
Female × male	F_1 genotypes (backcross): *a3/+; b1/+*	Other		coTE[b]
Wild type × *a3/a3; b1/+*	17	314		5.4 (male)
Wild type × *a3/+; b1/b1*	16	310		5.2 (male)
a3/a3; b1/+ × wild type	0	96		0 (female)
a3/+; b1/b1 × wild type	0	96		0 (female)

Transmission was defined as the number of plants from self-crosses having the mutant allele or wild-type allele, as determined by PCR using allele-specific primers for *XPO1A* and *XPO1B* alleles.

[a] (No. of mutant alleles/total no. of alleles) × 100 (%).

[b] coTE was defined as (no. of mutant plants/no. of wild-type plants) × 100 (Ebel *et al.* 2004).

TABLE 3

Percentage of aborted ovules in *xpo1*a/; *xpo1b* double mutants

Parental genotype	Normal	Aborted	Expected[a]	*P*-value[b]
+/+; +/+	1413	10 (0.7)	NA	NA
a1/a1; +/+	650	9 (1.4)	NA	NA
a2/a2; +/+	727	13 (1.8)	NA	NA
a3/a3; +/+	656	11 (1.6)	NA	NA
+/+; b1/b1	637	5 (0.8)	NA	NA
a3/+; b1/+	843	263 (24)	276.5 (25)	0.35
a3/a3; b1/+	620	573 (48)	596.5 (50)	0.17
a3/+; b1/b1	783	750 (49)	766.5 (50)	0.40
a1/+; b1/b1	352	371 (51)	361.5 (50)	0.48
a2/+; b1/b1	861	9 (1.0)	NA	NA
a2/a2; b1/+	857	10 (1.2)	NA	NA
a2/ a2; b1/b1	442	159 (28.5)[c]		
Rescue	Normal	Aborted	% expected[a]	% *R*[d]
a3/+; b1/+; XaG/−	808	385 (17.6)	12.5–25	60

Mature siliques from self-pollinated plants of the different genotypes were opened and scored for seed abortion. *a1*, *xpo1a-1*; *a2*, *xpo1a-2*; *a3*, *xpo1a-3*; *b1*, *xpo1b-1*; +, wild-type allele. NA, not applicable. Numbers in parentheses are percentages.

[a] Fraction of aborted seeds in the case of female gametophyte lethal mutation.

[b] Probability of χ^2 for a normal distribution with a degree of freedom of 1.

[c] Range of seed abortion per silique (3.8–60%) (only siliques that had elongated were recorded).

[d] Percentage of rescue, $R = 100 \times (25–17.6)/12.5$. Twenty-five percent abortion is expected in the case of no rescue and 12.5% abortion is expected in the case of full rescue. *XaG* represents the *XPO1A::GFP* transgene.

Genetic analysis of T-DNA insertion lines: To test whether *XPO1A* and *XPO1B* were functionally redundant, we attempted to construct double mutants. The *a1*, *a2*, and *a3* mutants were crossed, respectively, to *b1* and the F_1 generation was allowed to self-fertilize. The F_2 siblings from individual F_1 plants were genotyped using PCR. The expected frequency of double homozygous mutant plants in the F_2 population is 1:16. How-

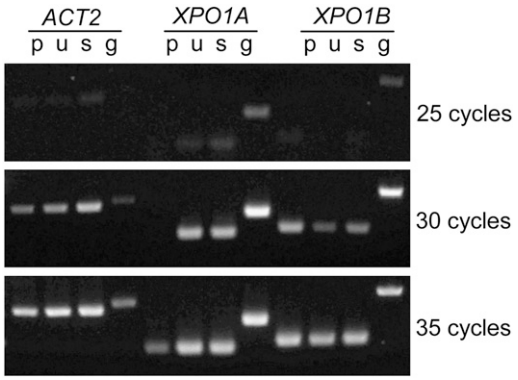

FIGURE 2.—Expression analysis of *XPO1*. RT–PCR analysis of *XPO1A* and *XPO1B* expression in p, pollen; u, unpollinated pistils; and s, seedling. *ACT2* was used as the control.

ever, a double homozygous mutant was not found in any of the screened F_2 populations ($n = 378$; Table 1), indicating segregation distortions. In addition, the proportion of plants homozygous at one locus and heterozygous at the other locus (HoHz) was significantly lower than expected from *a1/+; b1/+* or *a3/+; b1/+* F_1 plants, indicating that cotransmission of the mutant alleles through the gametes was impaired (Table 1). To increase the probability of finding double mutants, we screened the F_3 progeny of self-pollinated HoHz plants (Table 2). We could not find any *a1/a1; b1/b1* or *a3/a3; b1/b1* plants, but an *a2/a2; b1/b1* plant was obtained. Given that a transcript could be detected from *xpo1a-2* (Figure 1), the recovery of this genotype might be due to leaky expression from this allele. To determine if sporophytic or embryo lethality could account for the lack of double homozygotes, mature siliques of the different genotypes, including HoHz plants, were inspected for the presence of aborted ovules. The siliques of *a3/+; b1/+* doubly heterozygous plants had 25% undeveloped ovules, while siliques of the HoHz plants *a3/+; b1/b1* and *a3/a3; b1/+* had 50% undeveloped ovules. These results were consistent with a gametophytic mutation where female gametophytes are defective when the two mutant alleles are co-inherited (Table 3 and Figure 4, A and F). In addition, *XPO1a* was fused to GFP (XaG) and expressed in Arabidopsis under the control of the CaMV 35S RNA promoter. There were fewer (17.6%) undeveloped ovules in the double heterozygote *a3/+; b1/+* that was also hemizygous for the transgene (XaG/−), indicating that *XPO1a* was able to rescue 60% of the *a3 b1* embryo sacs. Whereas siliques of either HoHz (*a2/a2; b1/+* or *a2/+; b1/b1*) did not show significant ovule abortion, *a2/a2; b1/b1* plants showed reduced seed set, with 30% ovule abortion on average, indicating that, despite the presence of wild-type transcripts (Figure 1), *a2* is not equivalent to its wild-type allele in the *b1* background.

Siliques of the HoHz plant *a1/+; b1/b1* had 50% undeveloped ovules as in *a3/+; b1/b1*. Since *a1* and *a3* alleles showed the same phenotypic defect, we concluded that the 24-aa deletion in *a1* caused a loss of function. Because a defect was not observed in the single mutants, we concluded that *XPO1A* and *XPO1B* were functionally redundant.

***xpo1* double mutants show transmission defects through the male and the female:** To identify the cause of distorted segregation, cotransmission efficiencies (coTE) of mutant alleles through each gamete were

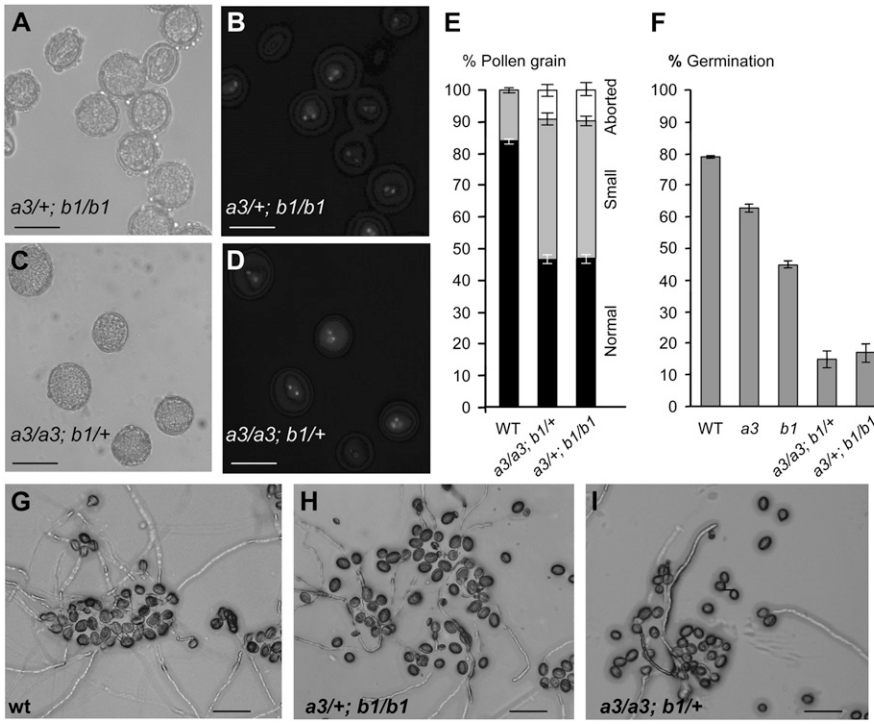

FIGURE 3.—Phenotype of *xpo1* mutant pollen grains. Transmitted light image of pollen grains from *a3/+; b1/b1* (A) and *a3/a3; b1/+* (C) plants. DAPI-stained pollen grains from *a3/+; b1/b1* (B) and *a3/a3; b1/+* (D) plants. (E) Size distributions of pollen in wild type (WT) and the *a3/+; b1/b1* and *a3/a3; b1/+* plants. (F) Percentage of germination, ±SE ($n = 3$) of pollen grains from wild type, *a3/a3*, *b1/b1*, *a3/+; b1/b1*, and *a3/a3; b1/+*. (G–I) Germination defect in *xpo1* mutant pollen grains. Representative images of germination assays with pollen from wild-type Col-0 (G), *a3/+; b1/b1* (H), and *a3/a3*; *b1/+* (I) plants. Bars, 30 μm (A–D) or 100 μm (G–I).

determined. The genotypes of the gametes should be equally distributed if segregating alleles are normally transmitted. Reciprocal crosses showed that the null alleles *a3* and *b1* could not be cotransmitted through the female when wild-type pollen was used to pollinate *a3/a3; b1/+* pistils or *a3/+; b1/b1* pistils (Table 2) and that cotransmission of the *a3* and *b1* alleles through the male was reduced to 5%. Therefore, the double-mutant pollen was partially functional, but the double-mutant embryo sac was not. These results indicate that *XPO1* is required for functional male and female gametophytes.

***XPO1* genes are expressed in male and female reproductive tissues:** We assessed *XPO1* expression during Arabidopsis development. Publicly available microarray data sets at GENVESTIGATOR (Zimmermann *et al.* 2004) showed that both transcripts were ubiquitously expressed in sporophytic tissues and that *XPO1A* was usually expressed at higher levels. However, in pollen microarray data sets (Becker *et al.* 2003; Pina *et al.* 2005), *XPO1A* was called absent and *XPO1B* was called present. To evaluate *XPO1* expression in the gametophytes, we tested the presence of both transcripts by RT–PCR, using RNA samples from mature pollen grains, unpollinated pistils, and 2-week-old seedlings. These results showed that both *XPO1A* and *XPO1B* are expressed in male and female structures, although their levels of expression were reciprocal. In unpollinated pistils, *XPO1A* was more highly expressed than *XPO1B*. *XPO1A* was detected in pollen but only after 35 cycles of PCR (Figure 2); sequencing confirmed that the product was *bona fide* and was not due to primer mis-annealing.

***xpo1* mutations affect pollen germination and tube growth:** To identify the defect in the mutant male gametophyte, the viability, morphology, and germination ability of pollen were analyzed in HoHz plants, in which 50% of the pollen is expected to be doubly mutant. A viability assay with Alexander's stain (Johnson-Brousseau and McCormick 2004) showed that 90.2% (*a3/a3; b1/+*, $n = 610$) or 88.9% (*a3/+; b1/b1*, $n = 135$) of the pollen grains were viable. In addition, all pollen had normal nuclear constitutions with two sperm nuclei and a vegetative nucleus (Figure 3, A–D). However, unlike wild type, 40% of the pollen grains were smaller and 10% were shriveled. The percentage of pollen grains showing morphological alterations was similar in *a3/+*; *b1/b1* and *a3/a3; b1/+* plants and correlated with the expected percentage of double-mutant pollen (Figure 3E). To assess the functional defects caused by the *xpo1* mutations, *in vitro* pollen germination of HoHz mutants, single mutants, and wild type were compared (Figures 3, F–I). The germination of single mutants was slightly reduced compared to wild type, but pollen germination of both HoHz mutants was strongly impaired. Moreover, pollen tubes of the small pollen grains were thinner and shorter than pollen tubes of normal-sized pollen grains. We therefore concluded that the affected pollen germination and tube growth could account for the genetic disadvantage reflected in the cotransmission deficiencies through the male.

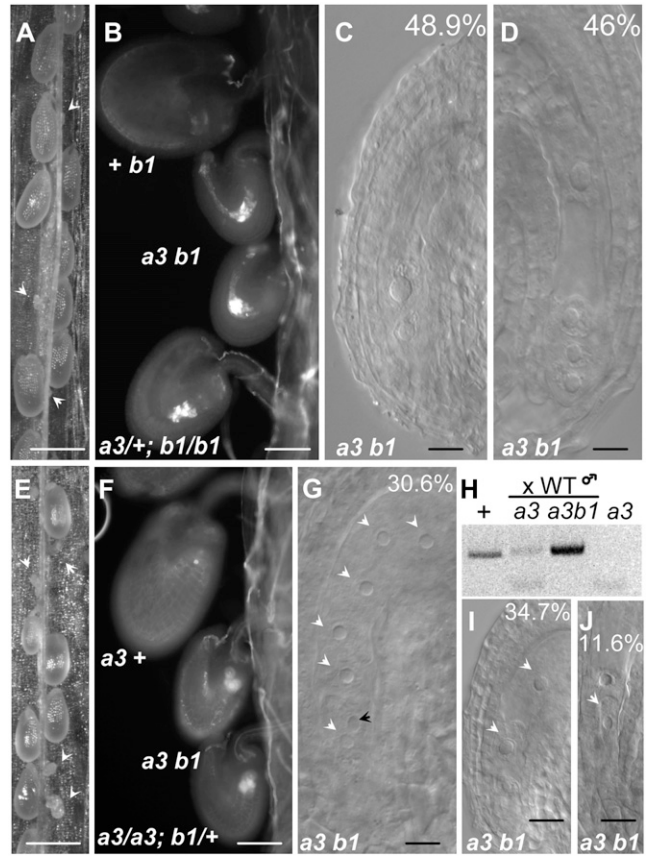

Figure 4.—Phenotype of *xpo1* mutant female gametophytes. (A–D) *a3/+; b1/b1*. (E–G, I, and J) *a3/a3; b1/+*. Open mature siliques; arrowheads indicate aborted or undeveloped ovules. (B and F) DAB-stained pistils 3 days after pollination. Genotypes are given for the female gametophyte. (C, D, G, I, and J) DIC images of whole-mounted cleared *a3/a3; b1/b1* ovules. Percentages represent the occurrence of the depicted phenotype. Arrowheads show endosperm nuclei in G and I or the zygote in J. (H) Single-ovule PCR to detect the paternal *XPO1A* allele, 3 days after pollination of *a3/a3; b1/+* pistils with wild-type (WT) pollen. Bars, 20 μm except in A and E (500 μm) and in B and F (100 μm).

***xpo1* mutations affect female gametophyte development:** To determine the nature of the defect in the female gametophyte, we analyzed pollen tube attraction and discharge by staining self-pollinated *a3/a3; b1/+* and *a3/+; b1/b1* pistils with DAB 3 days after pollination (Figure 4; supplemental Figure S2). Two distinct phenotypes were observed. In the *a3/+; b1/b1* plants (Figure 4, A–C), there were undeveloped ovules (presumably with embryo sacs that were *a3 b1*) that had failed to attract pollen tubes. In the *a3/a3; b1/+* plants, most ovules were able to attract a pollen tube (Figure 4F; supplemental Figure S2; Table 4). These results indicated that the phenotype of the embryo sac did not depend only on its genotype, but also on the genotype of the sporophyte, indicative of a female sporophytic influence on embryo sac development.

TABLE 4

Phenotypic distribution of *xpo1* mutant embryo sacs and pollen tube attraction

	Pollen tube attraction	*ab* from *a/+; b/b*	*ab* from *a/a; b/+*	*ab* from *a/+; b/+*
Two-nucleus ES	No	48.9% ($n = 86$)	0	0
Immature ES	No	46.6% ($n = 82$)	0	0
Unfertilized ES	No	0	10.2% ($n = 15$)	35% ($n = 42$)
Two-nucleus eM	Yes	0	47.6% ($n = 70$)	10.8% ($n = 13$)
eM proliferation (no Z)	Yes	0	30.6% ($n = 45$)	10.8% ($n = 13$)
Arrested Z (no eM)	Yes	4.5% ($n = 8$)	11.6% ($n = 17$)	23.3% ($n = 28$)
Arrested Z (with eM)	Yes	0	0	20% ($n = 24$)
n		176	147	120

ES, embryo sac; Z, zygote or pro-embryo; eM, endosperm; *n*, number of mutant ovules counted; normal ovules were not considered in the given percentages. Immature embryo sacs possessed the correct number of nuclei with an abnormal appearance.

We examined ovules from each HoHz plant and from HzHz plants (Figures 4 and 5; Table 4). About half of the *a3 b1* embryo sacs from *a3/+; b1/b1* plants showed arrested development at the two-nucleus stage (Figure 4C). The other *a3 b1* embryo sacs contained the expected number of nuclei, but cellularization was affected, the cells were not correctly positioned, and the nuclei were often larger (Figure 4D). Hence, *a3/+; b1/b1* plants produced defective *a3 b1* embryo sacs that were unable to attract pollen tubes. From the *a3/a3; b1/+* plants, ~10% of the *a3 b1* embryo sacs were undeveloped, slightly immature, and unfertilized, but most double-mutant ovules attracted pollen tubes and were apparently fertilized, since endosperm proliferation was observed (Figure 4, G–J). In 47.6% of the mutant ovules, the endosperm was arrested at a two-nucleus stage, and a zygote was observed in 12.9% of these ovules. In 11.6% of the ovules, there was no apparent endosperm. Since embryos were not observed beyond the pro-embryo stage (Figure 4J), we concluded that the zygotes had failed to divide. In 30.6% of the ovules, a zygote was not present but the endosperm had proliferated. To test if fertilization had occurred in undeveloped ovules and to rule out that endosperm proliferation was caused by autonomous development, we pollinated *a3/a3*; *b1/+* pistils with wild-type pollen. After pollination, we used PCR genotyping on single ovules to assay for the wild-type *XPO1A* allele in the defective ovules. Since this allele would be detectable only if transmitted from the pollen, the results of Figure 4H show that fertilization occurred, at least in some of the defective ovules. Double-mutant ovules from the HzHz plants showed similar phenotypes. The embryo sacs developed normally and attracted pollen tubes but again embryos did not develop after fertilization (Figure 5). On average, defects were observed at later stages of gametogenesis than those observed in HoHz plants (Table 4), suggesting that gene dosage influenced the strength of the sporophytic maternal effect.

We concluded that the *a3* and *b1* mutant alleles are not equivalent when maternally inherited in the mutant background of the reciprocal gene. Different defects in $a3^s$ *b1* ("^{s}" is used to designate female inheritance of the segregating allele) and *a3* $b1^s$ indicate a maternal effect for which the development and function of the embryo sac depend on the genotype of the sporophyte. Consequently, the two *XPO1* genes are not equally involved and, despite its lower transcript abundance in pistils, *XPO1B* appears to play a greater role during development of the female gametophyte. The genotype affected at the latest stage (*a3* $b1^s$) indicates that *XPO1* has a critical function during the transition from the zygote to the embryo, since development did not proceed after fertilization. As in maternal-effect embryo lethal mutations and similar to mutations affecting imprinted genes

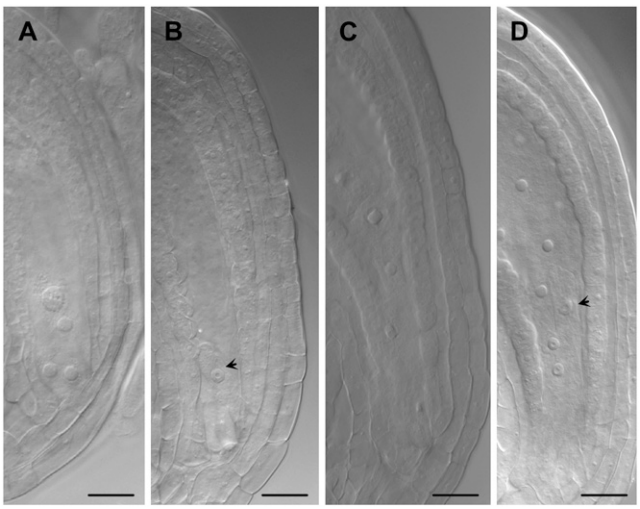

FIGURE 5.—Phenotype of *xpo1* mutant female gametophytes. Representative DIC images of *a3 b1* embryo sacs from a doubly heterozygous *a3/+*; *b1/+* plant (A), unfertilized embryo sac (B), zygote only (C), and endosperm only (D) arrested zygote with endosperm development. Arrowheads show the zygote. Bars, 40 μm.

(Chaudhury and Berger 2001), the paternal wild-type allele could not rescue the defective egg cell caused by lack of XPO1A and XPO1B.

DISCUSSION

Given the two defined functions of XPO1, the defects observed in *xpo1* mutants could be due to (i) the lack of export, causing nuclear contamination with factors that should be excluded from the nucleus; (ii) the lack of essential proteins or mRNAs in the cytoplasm; and/or (iii) malfunction of the kinetochores that would affect cell division. The correct development of the haploid gametophytes requires two mitoses for the pollen grain and three mitoses for the embryo sac. In yeast, the use of thermo-sensitive alleles of *crm1* showed that cells were immediately arrested at the next division following the switch to nonpermissive conditions (Adachi and Yanagida 1989). The scenario in the *xpo1* mutant plants seems slightly different, since the pollen grain can develop to maturity and the embryo sac can divide. Although a fitness disadvantage of the mutant pollen grains was observed, the mitotic divisions were not affected and the mature male gametophytes were partially functional. Fitness alterations of the mutant pollen may be due to impaired nuclear activities in the metabolically active vegetative cell or to a lack of exported proteins or mRNAs required for pollen tube germination and growth (Mascarenhas 1993). That mutant pollen grains are strongly affected in their ability to perform their functions supports the hypothesis that XPO1-dependent nuclear export is important in the male gametophyte but is not strictly required.

Defects in the female gametophyte were much stronger. The strongest defects were arrested nuclei within an embryo sac that did not complete the three rounds of mitosis. We suggest that a protein or mRNA pool of *XPO1* is carried over from female meiosis. This carryover might be sufficient to ensure the first steps of female gametogenesis. Therefore, the differential sporophytic maternal effect of the alleles, reflected in the strength of the phenotypes in the two HoHz and the HzHz, would be due to a dilution effect from different initial *XPO1* activities in the megaspore mother cell. Alternatively, one *XPO1* paralogous gene might be specific to diploid cells surrounding the embryo sac or might have a specialized function, such as a specific affinity for a cargo, which would be essential for female gametophytic development. In the latter scenario, it would not be the quantity but the quality of the carryover that would matter.

Mutant female gametophytes with milder defects attracted pollen tubes; the division of the endosperm, the presence of a zygote, and the detection of paternal alleles provided evidence of double fertilization. Perhaps the paternal allele rescued the endosperm or perhaps *XPO1* is not required for endosperm development. However, zygotes derived from mutant eggs aborted irrespective of the paternal contribution. Thus the *xpo1* mutation displays a maternal-effect lethality that acts much earlier than it does, for example, in *medea*, where the embryos abort at the late heart stage (Grossniklaus *et al.* 1998). In animals, during the maternal-to-embryonic transition, negative regulators of the Chromobox family members, including Polycomb factors and heterochromatin protein 1, are exported via the *Crm1/XPO1* pathway (Ruddock-D'Cruz *et al.* 2008). We hypothesize that *XPO1* could be required for proper function of the plant zygote through nuclear export of similar proteins. The developmental program that is established in the central cell requires at least some of the Polycomb factors in the nucleus, such as PRC2 (Guitton and Berger 2005). Thus endosperm development would be less affected by the absence of the export machinery and further divisions of the *xpo1* mutant endosperm would proceed. We suggest that XPO1 activity is necessary for the control of egg cell function. In failing to export some regulators, the mutant egg cell is affected in ways that compromise the development of the zygote.

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