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The Maize *aberrant pollen transmission 1* Gene Is a *SABRE/KIP* Homolog Required for Pollen Tube Growth

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ABSTRACT

Maize (*Zea mays*) pollen tubes grow in the styles at a rate of >1 μm/sec. We describe here a gene required to attain that striking rate. The *aberrant pollen transmission 1* (*apt1*) gene of maize was identified by an *Ac*-tagged mutation that displayed a severe pollen transmission deficit in heterozygotes. Rare *apt1* homozygotes can be recovered, aided by phenotypic selection for *Ac* homozygotes. Half of the pollen in heterozygotes and most of the pollen in homozygotes germinate short and twisted pollen tubes. The *apt1* gene is 26 kb long, makes an 8.6-kb pollen-specific transcript spliced from 22 exons, and encodes a protein of 2607 amino acids. The APT1 protein is homologous to SABRE and KIP, Arabidopsis proteins of unknown function involved in the elongation of root cortex cells and pollen tubes, respectively. Subcellular localization analysis demonstrates that APT1 colocalizes with a Golgi protein marker in growing tobacco pollen tubes. We hypothesize that the APT1 protein is involved in membrane trafficking and is required for the high secretory demands of tip growth in pollen tubes. The *apt1-m1(Ac)* mutable allele is an excellent tool for selecting *Ac* transpositions because of the strong negative selection pressure operating against the parental *Ac* site.

In higher plants, pollination is initiated with the transfer of pollen from the anther to the stigma. Once recognized by the stigma, the pollen hydrates and germinates a pollen tube. The pollen tube elongates along the transmitting tract in the style and delivers its two sperm cells to the female gametophyte to undergo double fertilization (McCormick 2004). Elongation of the pollen tube can be over a distance of a few hundred micrometers to several centimeters and constitutes the most rapid growth of any cell known (Hepler *et al.* 2001). This elongation is particularly remarkable in the polystigmatic styles of maize (*Zea mays*), where the pollen tube grows at the astounding rate of 1 cm/hr (Bedinger 1992). Evidence from a large collection of maize aneuploid stocks, in which development of the deficient microspores was slowed down or arrested, suggests that genes required for pollen development are expressed gametophytically and dispersed throughout the genome (Kindiger *et al.* 1991). By implication, mutations that affect male gametophytic development will be recovered rarely through the pollen. They can, however, be identified by the aberrant segregation of linked markers.

Several molecules that control pollen germination and the elongation and guidance of pollen tubes have been identified recently. Lipophilic molecules in the exine wall of the pollen (Lord 2000; Lord and Russell 2002), lipids (Preuss *et al.* 1993; Hulskamp *et al.* 1995a), pollen-coat proteins (Mayfield and Preuss 2000; Mayfield *et al.* 2001), and a calmodulin-binding protein (Golovkin and Reddy 2003) play important roles in pollen germination. Extracellular cues in the pistil and proteins produced by the embryo sac are likely involved in guiding the pollen tube toward the ovules and female gametophytes (Cheung *et al.* 1995; Hulskamp *et al.* 1995b; Ray *et al.* 1997; Shimizu and Okada 2000; Huck *et al.* 2003; Marton *et al.* 2005). Even the arrest of pollen tube growth by the embryo sac is under genetic control, as demonstrated by the *feronia* mutation in Arabidopsis, in which synergid degeneration fails to occur and pollen tubes continue to grow, leading to impaired fertility (Huck *et al.* 2003).

Substantial molecular, genetic, and cellular biological data have clearly demonstrated that pollen tube growth is tightly regulated by a Ca^{2+} gradient (Pierson *et al.* 1994; Holdaway-Clarke *et al.* 1997), a K^+ channel of the Shaker family (Mouline *et al.* 2002), a Ca^{2+}-dependent protein kinase (Estruch *et al.* 1994; Moutinho *et al.* 1998), an intact actin cytoskeleton (Chen *et al.* 2002, 2003), F-actin level (Fu *et al.* 2001), Rop GTPase (Li *et al.* 1999; Arthur *et al.* 2003), and Rab GTPase (Cheung *et al.* 2002). The protein products of

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two homologs, *SABRE* and the *SABRE*-like *KIP* (*KINKY POLLEN*), are required for the normal growth of Arabidopsis roots and pollen tubes, respectively (Aeschbacher *et al.* 1995; Procissi *et al.* 2003). *SABRE* affects root cortex cell expansion and appears to be expressed only in the sporophyte, whereas the *kip* mutation arrests the elongation of both pollen tubes and root hairs. The mechanism by which SABRE and KIP regulate cell expansion and tip growth, respectively, is not known.

We describe here a mutation with striking effects on pollen tube growth in maize. The mutation was uncovered by its aberrant pollen transmission in heterozygotes and named *aberrant pollen transmission 1* (*apt1*) (Xu and Dooner 2003). *apt1* is tagged by *Activator* (*Ac*), making it possible to use *Ac*'s *trans*-acting phenotype, the spotted seed color produced by a reporter allele, to follow its pollen transmission in maize. We show that pollen tube development and growth rate are severely arrested in the *apt1* mutant. The *apt1* gene, a homolog of *SABRE* and *KIP*, is extremely large for a plant gene: it extends over 26 kb, contains 22 exons, and makes an 8.6-kb transcript. Data from fluorescent APT1 fusion proteins point to a possible Golgi localization, suggesting a role for APT1 in vesicular trafficking during pollen tube elongation.

MATERIALS AND METHODS

Plant genetic stocks: All genetic stocks used in this study are in a W22 background. The *apt1* mutant was recovered in family 929.20 from an *Ac* insertion library derived from the *wx-m7(Ac)* mutable allele (Cowperthwaite *et al.* 2002). The *bz-m2(D1)* stock, which carries a *Ds* element in the second exon of the *bz* gene, was used throughout as *Ac* reporter and as the wild-type sibling control for the *apt1* mutant.

***In vitro* maize pollen germination:** Full, freshly dehisced anthers were collected from *apt1* heterozygous, homozygous, and wild-type plants. The pollen germination medium is made up of 12% sucrose, 0.03% calcium chloride, 0.01% boric acid, 0.4% DMSO, 0.6% Seakem LE agarose (Cambrex Bio Science, Rockland, ME). Pollen was gently squeezed from the anther and evenly dusted on the surface of a plate with germination medium. Plates were incubated in the dark at 25°. Pollen tubes were photographed at serial time points of 15 min, 30 min, 1 hr, 2 hr, 3 hr, and 4 hr on a dissecting microscope using bright field illumination. Pictures were saved as TIFF files in Adobe Photoshop and opened by an NIH imaging program (http://rsb.info.nih.gov/ij/docs/intro.html). Pollen tube length was then measured by tracing along the length of the tube in the picture. Pollen tubes were numbered to compare growth rates of individual tubes at different time points. Measurements were taken for 158 pollen tubes from three wild-type plants and for 176 pollen tubes from two *apt1* homozygous plants.

Chromosome mapping: Recombinant inbred lines from a cross between T232 and CM37 were used to the map the genetic location of *apt1* (Burr *et al.* 1988). Gels were blotted onto membranes and hybridized with a radiolabeled *tac929.20* fragment (*apt1* exons 14 and 15). Several restriction enzymes were tested on the parental genomic DNA and *Eco*RI gave the most distinct RFLPs. The segregation of RFLPs in recombinant inbred (RI) populations was scored and compared with the RI database from Brookhaven National Laboratory (http://www.maizegdb.org/cgi-bin/displayposrecord.cgi?id=41579).

Nucleic acid extraction and hybridization: Genomic DNA from seedlings and mature leaves was prepared by a urea extraction procedure (Greene *et al.* 1994). DNA blots and RNA blots were performed as previously described (Cowperthwaite *et al.* 2002).

PCR, BAC screening, and sequencing: PCR was performed according to the protocol of QiaTaq (QIAGEN, Valencia, CA). PCR products were cloned into pGEM-T Easy vector (Promega, Madison, WI) and transformed into DH5α competent cells. Plasmids were purified with a QIAGEN spin miniprep kit. DNA sequencing was carried out in an ABI 377 sequencer (Perkin-Elmer, Torrance, CA) following the manufacturer's instructions. The *tac929.20* fragment was isolated by inverse (I)PCR, which was optimized for isolation of *Ac tac* sites in the Dooner laboratory (Cowperthwaite *et al.* 2002). Two pairs of primers, which were designated as Ac2578R and Ac4473 and Ac2494R and Ac4542, were used for IPCR and a subsequent nested PCR. The sequences of the four oligonucleotide primers were as follows: Ac2578R, 5′-GACAAACATACC-TGCGAGGATCAC-3′; Ac4473, 5′-TCCCGTTTTCGTTTCCGTC-3′; Ac2494R, 5′-AC GCATCCCTCAACATCAAATAGG-3′; and Ac4542, 5′-CGTTA CCGACCGTTTTCATCCCTA-3′. BAC filters harboring CHORI *Mbo*I B73 BAC clones were screened with a *tac929.20* probe and a series of positive clones were obtained. The B73 BAC clone ZMMBBc0301K11 was selected for sequencing and was confirmed to contain the central and 3′ part of the *apt1* gene. Another B73 BAC clone, ZMMBBc0235P22, containing the 5′ and central part of the *apt1* gene and overlapping with the previous BAC clone, was identified among the maize FPC BAC contigs (http://www.genome.arizona.edu/fpc/maize/WebAGCoL/WebFPC/). By sequencing subclones from these two BAC clones, a complete *apt1* genomic sequence was obtained. The *apt1* genomic sequence has been deposited in GenBank under accession no. DQ020097.

Amplification of *apt1* cDNA: A partial *apt1* cDNA was initially amplified by RT–PCR. RT–PCR was performed according to the protocol of QiaTaq (QIAGEN, Valencia, CA). Total RNA extracted from W22 mature pollen was used as a RT–PCR template. Six overlapping pairs of primers were used to cover the full length of the cDNA: primers 5′-UTR-1 and apt 6423R, from the 5′-UTR to exon 8 (1.5 kb); primers apt 6218F and apt 14054R, from exon 8 to exon 9 (2.5 kb); primers xbaI-7F and xbaI-12R, from exon 9 to exon 10 (2.0 kb); primers xbaI-11F and apt 18109R, from exon 10 to exon 12 (1.0 kb); primers con7-5′ and apt 23888R, from exon 12 to exon 19 (1.0 kb); and primers con4-5′ and BACEcorI3′-1R, from exon 18 to exon 22 and the 3′-UTR (1.5 kb). The sequences of the 12 oligonucleotide primers are provided in supplemental Table 1 (http://www.genetics.org/supplemental/). RT–PCR products were cloned into pGEM-T Easy (Promega) and then sequenced on an ABI 377 sequencer (Perkin-Elmer). An *apt1* full-length cDNA was constructed and fused to the GFP N terminus (see below). The *apt1* transcript sequence has been deposited in GenBank under accession no. DQ020098.

GFP and DsRed fusion protein construction: A pLAT52–GUS vector was kindly provided by Sheila McCormick. The fragment containing the LAT52 promoter was released by *Apa*I and *Nco*I digestion from pLAT52–GUS and then cloned into pGEM-T Easy (Promega). This pLAT52–GEM-T Easy vector contains an ATG translation start codon at the *Nco*I site. An alanine linker with *Kpn*I and *Nco*I adapter sites was cloned into the multiple cloning site of a plant GFP expression vector, pFF–GFP, kindly provided by Gregorio Segal. The GFP–35s terminator segment of pFF–GFP was released by *Nco*I and *EcoR*I digestion and used to replace the GUS–NOS terminator segment of pLAT52–GUS, yielding pLAT52–GFP–35sT. The

APT1 S4 fragment, which covers the *apt1* transcript from site 7055 to 7533, was amplified with primers *Sac*II 7055F and *Kpn*I 7533R. The S4 fragment was digested with *Sac*I and *Kpn*I and cloned, together with the pFF–GFP fragment alanine linker–GFP–35s terminator (released by *Kpn*I and *Eco*RI), into *Sac*II–*Eco*RI double-digested LAT52–pGEM-T Easy in a three-way ligation. We call the resulting construct pLAT52–S4–Ala–GFP. This construct was used as the backbone for the following GFP fusion constructs: R1R6, R2, CHO1, CHO2, CHO3, CHO4, S2, S5, and N6, as described in the supplemental Materials and Methods (http://www.genetics.org/supplemental/).

The DsRed coding sequence was amplified from the pDsRed2–C1 vector (CLONTECH, Palo Alto, CA) by PCR with a high-fidelity polymerase *rTth* XL. A pair of *Sac*II- and *Sph*I-adapted primers, RedC1-5′SacII and RedC1-3′SphI, was used for the amplification. The DsRed fragment was digested with *Sac*II and *Sph*I and then cloned into the pLAT52–S4–Ala–GFP backbone by replacing the S4–Ala–GFP fragment. A pLAT52–DsRed–35sT vector was produced, with the multiple cloning sites at the C terminus of DsRed. The c1BK1, c1Bk2, and c1BK3 fusions to DsRed were constructed as described in supplemental Materials and Methods (http://www.genetics.org/supplemental/).

The *GmManI* coding region was amplified from the GmManI::GFP fusion vector pBP30, kindly provided by Andreas Nebenführ. A pair of *Bgl*II- and *Kpn*I-adapted primers, designated as Man-BglII5′F and Man-KpnI3′R, was used for this amplification. The amplified *GmManI* fragment was digested with *Bgl*II and *Kpn*I and then cloned into the pLAT52–DsRed–35sT vector at the multiple cloning site, producing pLAT52–DsRed–GmManI. The sequences of the alanine linker primers are: top, 5′-CAAGCTTGCTGCCGCACCTG TCGCGGCAGCCGCTGCGGC-3′; bottom, 5′-CATGGCCGCA GCGGCTGCCGCGACAGGTGCGGCAGCAAGCTTGGTAC-3′. T4 PNKase-treated oligos were denatured and then annealed to form a short fragment for the cloning described above. Sequences of the other primers are provided in supplemental Table 1 (http://www.genetics.org/supplemental/).

Microprojectile bombardment of tobacco pollen: The biolistic particle delivery system PDS-1000 (Bio-Rad Laboratories, Hercules, CA) was used for transient expression of GFP and DsRed marker in tobacco pollen. Gold particle preparation and bombardment procedure followed a previously reported protocol in Z. Yang's laboratory (Fu *et al.* 2001). Colocalization analysis of GFP and DsRed markers was accomplished with mixing both GFP and DsRed constructs in one bombardment shooting.

RESULTS

The *apt1* mutation is defective in *Ac* pollen transmission: *Ac* segregates aberrantly in the self progeny of plants carrying *Ac929.20*, one of a collection of *transposed Ac* (*trAc*) elements derived from *wx-m7(Ac)* (Cowperthwaite *et al.* 2002). The *bz* mutable allele *bz-m2(D1)*, which harbors a *Dissociation* (*Ds*) element in the *bz* gene, was used as a reporter of *Ac* activity. In the presence of *Ac*, this reporter produces an unstable aleurone color phenotype consisting of purple spots on a bronze background (bz-m); in its absence, it produces a solid bronze phenotype (bz). Because *Ac* generally has a negative dosage effect, the degree of spotting is inversely correlated with the dosage of *Ac* in the genome. One dose of *Ac* produces a coarse spotting pattern (bz-mC) and additional doses lead to increasingly finer spotting. Thus, it is possible to score the activity and dosage of *Ac* in each individual kernel of a segregating ear. In the self progeny of transposant 929.20 the segregation ratio of *Ac* to no *Ac* was 1:1, instead of 3:1, and very fine spotted kernels, indicative of *Ac* homozygotes, were rare. Yet, the mutant ear had a normal seed set, suggesting that the *trAc* had caused a mutation defective in pollen, but not egg, viability. The genetic analysis described below confirmed that the mutation had been caused by an *Ac* insertion, so we designated the affected gene *apt1* for *a*berrant *p*ollen *t*ransmission *1* and the new mutant allele *apt1-m1(Ac)*.

Table 1 shows the analysis of *Ac* transmission in *apt1-m1(Ac)*/+ heterozygotes. Self progenies of *apt1-m1(Ac)*/+; *bz-m2(D1)* segregated 353 bz-m:364 bz, which means that the ratio of kernels with and without *Ac* was 1:1, instead of the expected 3:1. Testcross progenies of *apt1-m1(Ac)*/+; *bz-m2(D1)* as the maternal parent produced 1295 bz-m and 1277 bz, *i.e.*, the expected 1:1 ratio of kernels with and without *Ac*. Therefore, the female transmission of *Ac* is normal. The reciprocal cross using *apt1-m1(Ac)*/+; *bz-m2(D1)* as the paternal parent produced a total of 305 bz-m and 3797 bz, *i.e.*, a 1:12 ratio of kernels with and without *Ac*. Therefore, the male transmission of *Ac* is defective. Representative ears from these reciprocal crosses are shown in Figure 1: In the ear from the cross using the *apt1-m1(Ac)*/+ parent as female and the *bz-m2(D1)* reporter as male (Figure 1A), half of the kernels are spotted, as expected, whereas in the ear from the reciprocal cross (Figure 1B), very few kernels are spotted, indicating that *Ac* is transmitted poorly by the pollen parent.

The *apt1* mutation is caused by an *Ac* insertion: To verify that *apt1* cosegregated with *Ac*, DNA blots of spotted and nonspotted segregants from the testcross progeny of the *apt1-m1(Ac)*/+ female parents were hybridized with the central part of the *Ac* element. Figure 2 shows the result of hybridizing a blot containing *Sac*I-digested DNA from representative individuals. A unique band, indicated with an arrow, is present only in DNA from spotted seeds (Figure 2A). A similar result was obtained with *Eco*RI-digested DNA (not shown). The unique band was present in the 60 spotted segregants and absent from the 20 nonspotted segregants analyzed. These results confirm that *apt1* is linked with an *Ac*-hybridizing band. The fragment adjacent to the *Ac* insertion site was isolated by IPCR, using as a template *Eco*RI-digested, size-fractionated DNA from *apt1-m1(Ac)*/+ heterozygotes and primers based on *Ac* (Cowperthwaite *et al.* 2002). The amplified products contained a sequence with a perfect match to the 3′ end of *Ac* plus 1.2 kb of putative *tac929.20* sequence. The original DNA blots were rehybridized with the putative *tac929.20* site and the results for the *Sac*I digest are shown in Figure 2B. The *apt1-m1(Ac)*/+ heterozygous plants are also heterozygous for a band that is 4.6 kb

TABLE 1

***Ac* transmission analysis in *apt1(Ac)/+* heterozygotes**

Type of mating	Genotype of parents in mating	Seed phenotypes[a] bz-m	bz	*Ac*:no *Ac* ratio
Self	*apt1(Ac)/+; bz-m2(D1)* × same	353	364	1:1
Ac as female	*apt1(Ac)/+; bz-m2(D1)* × + ; *bz-m2(D1)*	1295	1277	1:1
Ac as male	+ ; *bz-m2(D1)* × *apt1(Ac)/+; bz-m2(D1)*	305	3797	1:12

[a] bz-m, spotted bronze kernel phenotype resulting from the presence of *Ac* in the genome; bz, uniformly bronze kernel phenotype resulting from the absence of *Ac*.

larger than the single band found in wild type, thus validating the *tac929.20* site and showing that it is present in single copy in the maize genome. The *tac929.20* site was mapped with RI lines (Burr *et al.* 1988) to position 78 in the long arm of chromosome 9 (bin 9.04), 23 cM away from *wx*. The RI mapping result is consistent with the placement of *Ac* to a location linked to *wx* in the F_2 progeny of a *Wx apt1-m1(Ac)/ wx* + heterozygote (Cowperthwaite *et al.* 2002).

To confirm that *apt1* was tagged by *Ac*, the spotted seed progeny of *apt1-m1(Ac)/+* heterozygotes was screened for restoration of *apt1* gene function following transposition of *Ac* to a new location in the genome. *Apt1′* revertants were sought first without selection among the spotted F_2 progeny of an *apt1-m1(Ac)/+* heterozygote. Of 36 individuals tested for their ability to transmit *Ac* as male parents, 35 resembled the *apt1-m1(Ac)/+* parent in transmitting *Ac* poorly, but one transmitted *Ac* normally. DNA blot analysis of this putative revertant showed that it had two *Acs*, one at the original *apt1-m1(Ac)* location and another one at a new location (Figure 3A). Subsequent genetic analysis revealed that this individual carried a *trAc* 6 cM away from a revertant *Apt1′* allele containing a typical 8-bp footprint (data not shown). As is discussed later, the extra 8 bases do not cause a mutant phenotype because *Ac* is inserted in an intron of the *apt1* gene. The presence of the 8-bp footprint allows one to distinguish the *Apt1′* revertant allele from the wild-type allele. Primers flanking the *Ac* insertion site were used to analyze the genotypes of the progeny from a cross between a wild-type female and the *Apt1′/apt1-m1(Ac), trAc/+* heterozygous revertant as male. PCR with these primers will amplify the wild-type and *Apt1′* revertant alleles, but not the *apt1-m1(Ac)* mutant allele. Figure 3B shows that the revertant (lane P1) produces a larger band than wild type (lane P2). The progeny of the cross using the revertant as male are all heterozygous for an *Apt1′* revertant allele and a wild-type allele. So, loss of *Ac* from the *apt1-m1*(*Ac*) allele appears to restore normal male transmission. DNA blot analysis (Figure 3C) showed that all the bz-m (*i.e.*, *Ac*-carrying) progeny of the cross had the *trAc* band, but not the *apt1-m1(Ac)* band. Therefore, the *trAc*, which is closely linked to the *Apt1′* revertant allele, is male transmitted, but the *Ac* in the *apt1-m1(Ac)* mutant allele is not. From the above reversion analysis we conclude that an *Ac* insertion caused the *apt1* mutation.

The *apt1-m1(Ac)* allele can be occasionally transmitted by the pollen: Given the selective disadvantage of *apt1-m1(Ac)* pollen in *apt1-m1(Ac)/+* heterozygotes, it is likely that many of the *Ac* progeny of an *apt1-m1(Ac)/+* pollen parent will carry a *trAc* element rather than the one originally found at the *apt1* locus. To test this prediction, 16 bz-m progeny seeds of the cross between a +; *bz-m2(D1)* tester female and an *apt1-m1(Ac) /+; bz-m2(D1)* heterozygous male were analyzed. Figure 4 shows the result of hybridizing a blot containing *Sac*I-digested DNA with an *Ac* probe. An *apt1-m1(Ac)* unique band (confirmed with the *tac929.20* probe, data not shown) is present in the male parent (lane P1). All 16 bz-m progeny carry a *trAc*

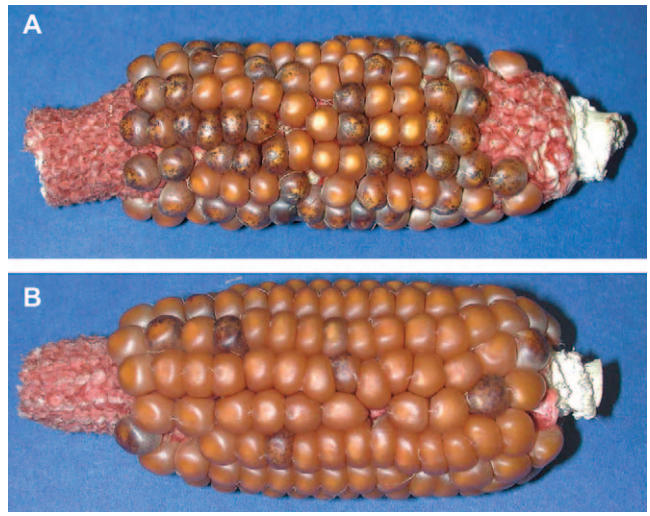

Figure 1.—Ears from reciprocal crosses between *apt1-m1(Ac)/+; bz-m2(D1)* × +; *bz-m2(D1)* individuals, showing *Ac* transmission in the female and male gametophytes. (A) An ear using the *apt1-m1(Ac)/+* parent as female. The bz-m and bz kernel phenotypes segregate in a 1:1 ratio, indicating that *Ac* transmission by the female gametophyte is normal. (B) An ear of the reciprocal cross using the *apt1-m1(Ac)/+* parent as male. The bz-m and bz kernel phenotypes segregate in a 1:12 ratio, indicating that *Ac* transmission by the male gametophyte is defective.

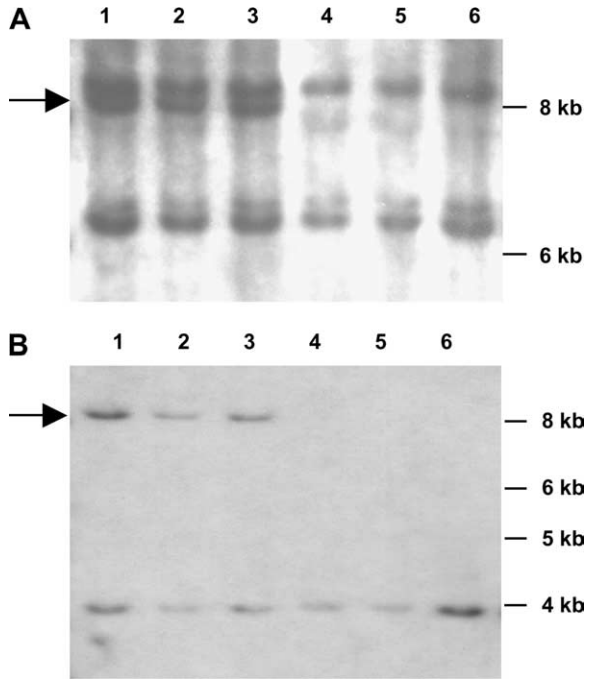

FIGURE 2.—DNA blot showing that the *apt1* mutation cosegregates with *Ac*. Genomic DNA from spotted (lanes 1–3) and nonspotted (lanes 4–6) testcross segregants was digested with *Sac*I. (A) Filter hybridized with an *Ac* internal probe. (B) The same filter hybridized with a *tac929.20* probe. The arrow shows the unique band common to both panels.

band (marked by an arrowhead), but only the individual in lane 1 carries a parental *apt1-m1(Ac)* band. Therefore, only 1 of 16 *Ac* selections (~6%) carries the *apt1-m1(Ac)* allele. The other selected individuals carry *trAc*s. An additional 120 bz-m selections from the same cross were planted, and the selections were either selfed or crossed as males to wild type. All of these progenies showed normal transmission of *Ac*, indicating that *Ac* had transposed from *apt1*. We conclude that *apt1-m1(Ac)* is only rarely male transmitted. On the basis of the *Ac* male transmission data in Table 1 and the results of the above DNA blot analysis, the male transmission ratio of the *apt1-m1(Ac)* chromosome relative to its normal homolog is estimated to be ~5:1000 [(305/3797) × (1/16)].

***apt1* homozygous mutants exhibit short and twisted pollen tubes and strong selection for reversion events:** Because *apt1-m1(Ac)* can be occasionally male transmitted, we set out to recover *apt1-m1(Ac)* homozygotes, which would enable us to examine the sporophytic effects of the mutation. From self matings of 150 *apt1-m1(Ac)/+; bz-m2(D1)* plants, 120 fine-spotted (bz-mF) seeds were obtained among 22,500 seeds. These bz-mF seeds should carry more than one copy of *Ac* and are, therefore, homozygous *apt1-m1(Ac)* candidates. Seventy-eight of these 120 bz-mF individuals were genotyped by DNA blotting and 4 of them turned out to be homozygous *apt1-m1(Ac)* mutants.

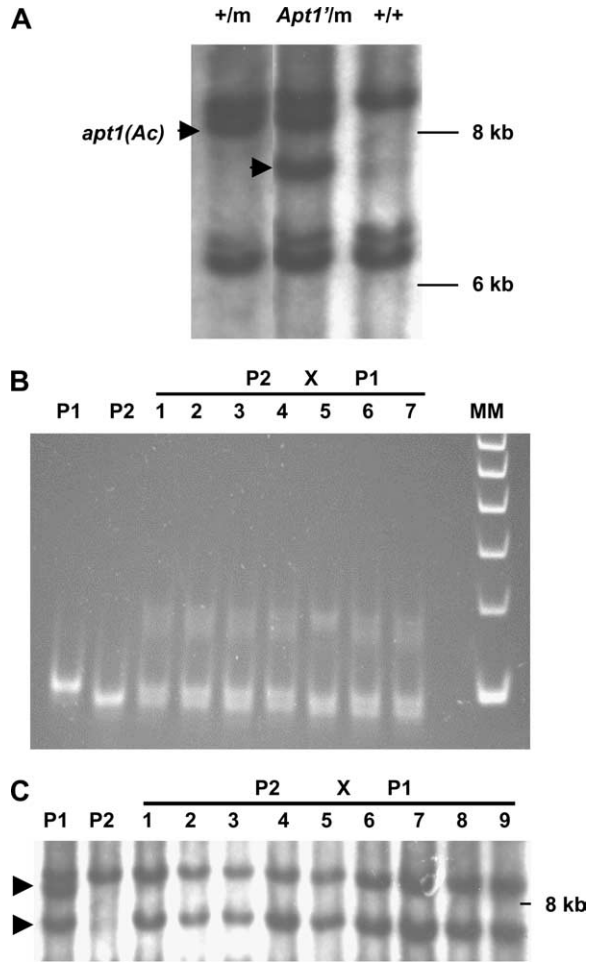

FIGURE 3.—*Ac* excision restores the pollen transmission of *Apt1′* revertant alleles. (A) DNA blot analysis of an *Apt1′* revertant. The revertant has two *Ac*s (indicated with arrowheads): One is the *Ac* element in *apt1*, and the second one is a *trAc*. Genomic DNA was digested with *Sac*I and hybridized with an *Ac* internal probe. Lanes: +/m, +/*apt1-m1(Ac)* heterozygote; *Apt1′*/m, *Apt′*/*apt1-m1(Ac)* heterozygous revertant; +/+, wild type. (B) Pollen transmission of the *Apt1′* excision allele. Wild-type and revertant alleles were PCR amplified using *apt1* primers flanking the *Ac* insertion site and the PCR products were separated in an 8% PAGE gel. P1, *Apt1′*/*apt1-m1(Ac)* heterozygous revertant parent (the mutant allele fails to amplify because of the 4.6-kb *Ac* insertion); P2, wild type; lanes 1–7, randomly selected progenies from the cross between a P2 female and a P1 male (all are heterozygous for the fragments amplified in the parents); MM, 100-bp DNA ladder. The band in P1 is 8 bp larger than that in P2, as determined by sequencing. (C) DNA blot analysis of the pollen transmission of the two *Ac*s present in the *Apt1′* revertant. Genomic DNA was digested with *Sac*I and the blot was hybridized with an *Ac* probe. The *Ac* in *apt1* (top band) is not male transmitted, but the *trAc* (bottom band) is. P1, *apt1-m1(Ac)*/*Apt1′* +/*trAc* double heterozygote; P2, wild type; lanes 1–9, bz-m progenies of the cross between a P2 female and a P1 male.

Mature pollen from *apt1-m1(Ac)* homozygous, heterozygous, and wild-type plants was collected and germinated *in vitro*. Compared with wild type, the *apt1-m1(Ac)* homozygous mutant shows mostly short and twisted pollen tubes (Figure 5, A and C). Sporadically, however,

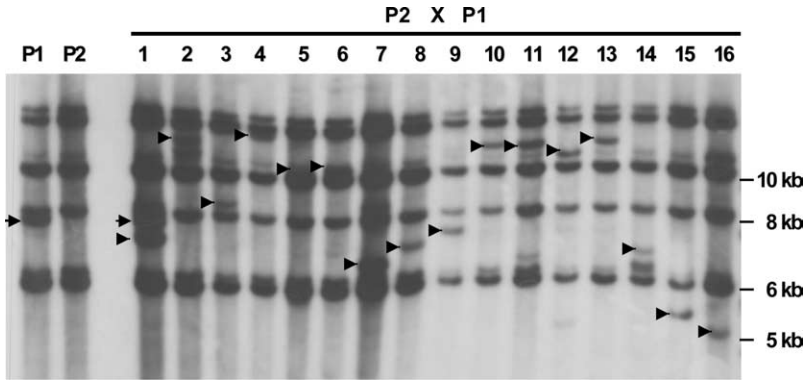

FIGURE 4.—DNA blot analysis of pollen-transmitted *Ac* elements from *apt1-m1(Ac)/+*. Genomic DNA from *apt1-m1(Ac)/+* heterozygotes (lane P1), wild type (lane P2), and randomly selected spotted seed (*i.e.*, carrying *Ac*) of the cross between a P2 female and a P1 male (lanes 1–16) was digested with *Sac*I and hybridized with an *Ac* internal probe. The arrowhead in each lane indicates a unique *trAc* band; the full arrow indicates the parental *apt1-m1(Ac)* band.

the mutant also shows some long and normal pollen tubes, which are most likely *Apt1′* revertants arising from *Ac* excision (see previous section). For example, in the plating of pollen from one anther, 4 out of 77 germinated pollen tubes appeared normal. This is a manifestation of the mutable phenotype of the *apt1-m1(Ac)* homozygous mutant. The heterozygous *apt1-m1(Ac)/+* plants show a 1:1 segregation of wild-type and mutant pollen tube phenotypes (Figure 5B). The gross appearance of mature pollen grains was examined with a portable 10× field microscope, used routinely to score pollen abortion in deficiency and translocation heterozygotes, but no difference could be seen among the pollen grains of an *apt1-m1(Ac)/+* heterozygous plant. The growth rates of wild-type and mutant pollen grains were compared by *in vitro* germination tests. As shown in Figure 6, wild-type pollen tubes grow dramatically faster than mutant pollen tubes (255 μm/hr *vs.* 34 μm/hr), suggesting that the mutant pollen tube's competitive ability in heterozygotes is severely compromised.

Analysis of the self progenies of *apt1-m1(Ac)* homozygous mutants reveals a strong selection for *Ac* excision products. In one self-pollinated ear from an *apt1-m1(Ac) bz-m2(D1)* homozygote, roughly equal numbers of bz-mF and bz-mC seeds were obtained, rather than the bz-mF majority expected from an *Ac* homozygote. The coarse spotted phenotype suggested that the bz-mC progeny were *Ac* heterozygotes and, in fact, all 23 individuals tested showed a heterozygous, *i.e.*, revertant, pollen tube phenotype in *in vitro* germination tests (1:1 ratio of long and short pollen tubes). Twenty-four bz-mF progenies were genotyped by DNA blots, using an *apt1* probe: 22 of them were *Apt′/apt1-m1(Ac)* heterozygous revertants and only 2 were *apt1-m1(Ac)* homozygotes (data not shown). The latter 2 progenies showed a homozygous mutant pollen tube phenotype and, again, evidence for strong selection of male gametes carrying *Ac* excisions. Maintaining the homozygous *apt1-m1(Ac)* mutant stock is not easy. Homozygous *apt1-m1(Ac)* individuals must first be screened on the basis of *Ac*'s dosage effect on the

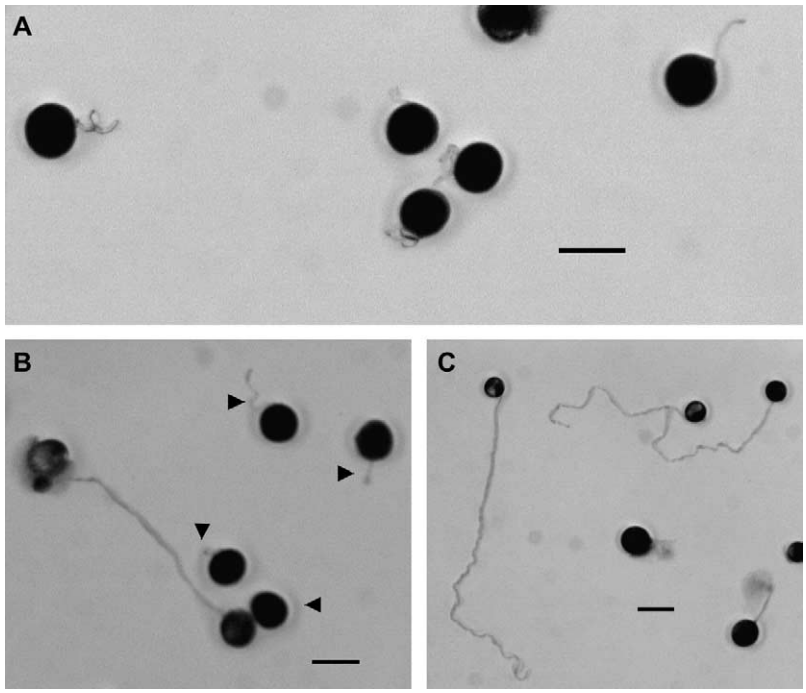

FIGURE 5.—Phenotypes of pollen tubes germinated *in vitro*. (A) Pollen from an *apt1* homozygous mutant grown for 4 hr. (B) Pollen from an *apt1/+* heterozygote grown for 4 hr. Arrowheads show the pollen tubes originating from mutant pollen grains. Compared to wild type, the *apt1* pollen tubes are short and twisted. (C) Wild-type pollen grown for 4 hr. Some wild-type pollen tubes have burst over this time period. Bars, 150 μm.

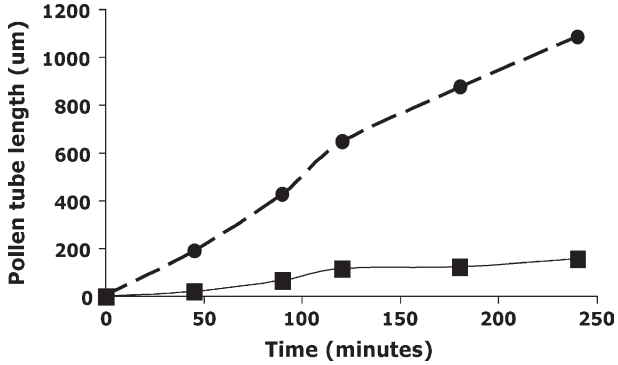

FIGURE 6.—Growth rate comparison of wild type (●) and *apt1* mutant (■) pollen tubes germinated *in vitro*. See MATERIALS AND METHODS for experimental details.

spotted-seed phenotype and, then, genotyped by DNA blots. Only a small fraction of *apt1-m1(Ac)* homozygotes are obtained. The strong tendency of the *apt1-m1(Ac)* mutant to revert provides further confirmation that the mutation is *Ac* tagged and indicates that the revertant pollen easily outcompetes the mutant pollen in the styles, thus preferentially transmitting its sperms to the next generation.

The *apt1* mutation does not appear to affect the sporophyte. The aerial parts of homozygous *apt1-m1(Ac)* plants show no difference from wild type, even in fast tip-growing tissues like the silks. We did not see differences between the roots of mutant and wild-type seedlings, but did not attempt to examine the roots of mature plants after the homozygotes had been transferred to soil.

The protein encoded by *apt1* is homologous to the SABRE and KIP proteins in Arabidopsis: As reported earlier (COWPERTHWAITE *et al.* 2002), the amino acid sequence encoded by the *tac929.20* site shows homology to SABRE in Arabidopsis, which has been annotated as a very large protein (~2600 aa). Because isolation of the complete progenitor wild-type allele of the *apt1-m1(Ac)* mutation from the W22 inbred would have been difficult and time consuming, we decided to take advantage of a maize public B73 BAC library to clone the *Apt1-B73* allele, instead. Two overlapping BACs were identified that contained the entire *apt1* gene. First, BAC clone ZMMBBc0301K11 was isolated using a *tac929.20* probe and, then, the contiguous BAC ZMMBBc0235P22 clone was identified from the maize FPC map (http://www.genome.arizona.edu/fpc/maize/WebAGCoL/WebFPC/). Subclones covering 31 kb across these two BACs, and expected to contain the entire *apt1* gene, were made and sequenced. As anticipated, the *apt1* gene is very large, extending over 26 kb from one end to the other. A comparison of the sequences of *apt1* and *tac929.20* with those in the GenBank database revealed that *Ac* had inserted in a gene encoding a protein highly similar to SABRE and KIP from Arabidopsis (AESCHBACHER *et al.* 1995; PROCISSI *et al.* 2003) and to related, but not annotated, proteins from rice. Figure 7 diagrams the structure of the *apt1* gene and the site of insertion of *Ac* in the mutant. The *apt1* gene has 22 predicted exons and is bordered by a retrotransposon sequence ~1.5 kb upstream of the putative start codon and by another gene 2 kb downstream of the putative stop codon (not shown).

The *apt1* cDNA was amplified in overlapping segments by RT–PCR, using total RNA of W22 mature pollen as template. A full-length cDNA sequence was then constructed from the overlapping RT–PCR amplification products and verified by sequencing. The mature transcript measures 8.6 kb and is made up of 22 exons. Its coding region is 7821 nucleotides long and encodes a 2607-amino-acid protein that is homologous to the SABRE and KIP proteins from Arabidopsis and to two related proteins in rice (supplemental Figure 1 at http://www.genetics.org/supplemental/). The five proteins are very conserved throughout their lengths and share homology with other unknown yeast and animal proteins in the central and C-terminal segments. The C terminus of these proteins is the most highly conserved and may define a novel class of proteins, possibly targeted to the Golgi (see below).

The *apt1* gene is expressed in pollen only: To characterize the expression pattern of *apt1*, total RNA from different wild-type tissues was analyzed by RNA blots, using *tac 929.20* as probe (*tac929.20* carries exons 14 and 15; see Figure 7). The *apt1* gene appears to be expressed only in mature pollen (Figure 8A, lane 11; the weak signal detected in the mature tassel sample in lane 5 most likely originates from the pollen). On the basis of the RNA markers in the blot, the transcript size is estimated to be ~8 kb, in agreement with the length of the *apt1* cDNA. No signal could be detected in root, stem, leaf, immature tassel, immature ear, endosperm, or silk, even though the RNA from some of these tissues was overloaded relative to the pollen (Figure 8B).

The APT1 protein colocalizes with Golgi-targeted proteins in tobacco pollen tubes: To investigate the

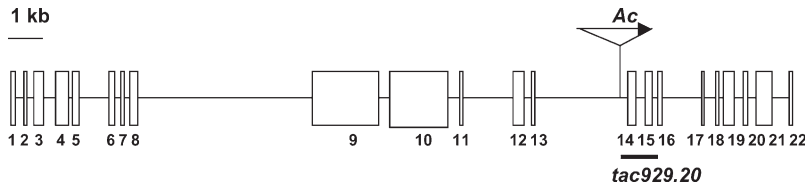

FIGURE 7.—Structure of the *apt1* gene. A diagram of the *apt1* genomic sequence is shown. The boxes stand for exons and the intervening lines for introns. In the mutable allele *apt1-m1*, *Ac* is inserted in intron 13 in the same transcriptional orientation as *apt1* (arrow). The location of the *tac929.20* probe (COWPERTHWAITE *et al.* 2002) is indicated with a bar.

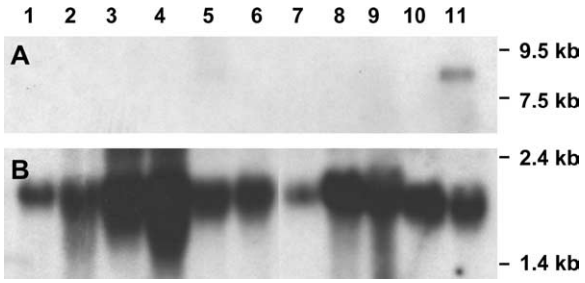

FIGURE 8.—RNA blot analysis of *apt1* expression in various tissues. (A) Blot hybridized with the *tac929.20* probe. (B) Same filter as in A rehybridized with an F-actin probe after removing the first probe. Lane 1, mature root; lane 2, stem; lane 3, young leaf; lane 4, mature leaf; lane 5, mature tassel; lane 6, immature tassel; lane 7, mature ear; lane 8, 14-day-old endosperm; lane 9, 35-day-old endosperm; lane 10, unpollinated silk; lane 11, mature pollen.

subcellular localization of the APT1 protein and, thus, obtain some indication of its possible function, several translational fusions of APT1 to a fluorescent protein were transformed into tobacco pollen tubes by particle bombardment. All the constructs are driven by the tomato LAT52 pollen promoter (TWELL *et al.* 1989) and carry either the green fluorescent protein GFP or the red fluorescent protein DsRed (Figure 9A). Figure 9B shows a schematic diagram of the different parts of the APT1 protein that were fused to the fluorescent protein markers. Details of the constructs are provided in MATERIALS AND METHODS.

GFP was fused to the C terminus of either the entire APT1 protein (R1R6) or a part thereof (R2, CHO1, CHO2, CHO3, CHO4, S2, S4, S5, and N6). In control experiments with GFP alone, GFP was usually distributed throughout the whole pollen tube (data not shown). In the bombardment experiments with APT1–GFP fusion constructs, R2, CHO1, and S4 produced diffusible signals like the GFP control. S2, S5, and N6 generated targeted signals in growing tobacco pollen tubes (Figure 10, A–C, and data not shown). The rest failed to produce a GFP image, possibly because of degradation of the fusion protein. To confirm the protein targeting of N6, S2, and S5, another type of construct was made, in which the N6 region was either extended or shortened and fused with DsRed at the N terminus (Figure 9B: c1Bk1–c1Bk3). In pollen bombardment experiments, the c1Bk1 and c1Bk2 constructs produced clear DsRed signals with the same distribution as the GFP signal of N6, S2, and S5 (data not shown). The c1Bk1 construct is the DsRed counterpart of S2, whereas the c1Bk2 construct shares the right end of c1Bk1, but is shorter than N6 at the left (N) end. Taken together, these results indicate that the C terminus of the APT1 protein contains signals for subcellular targeting. Specifically, the results with the S5 construct show that the region can be narrowed down to a 108-amino-acid stretch in the C terminus.

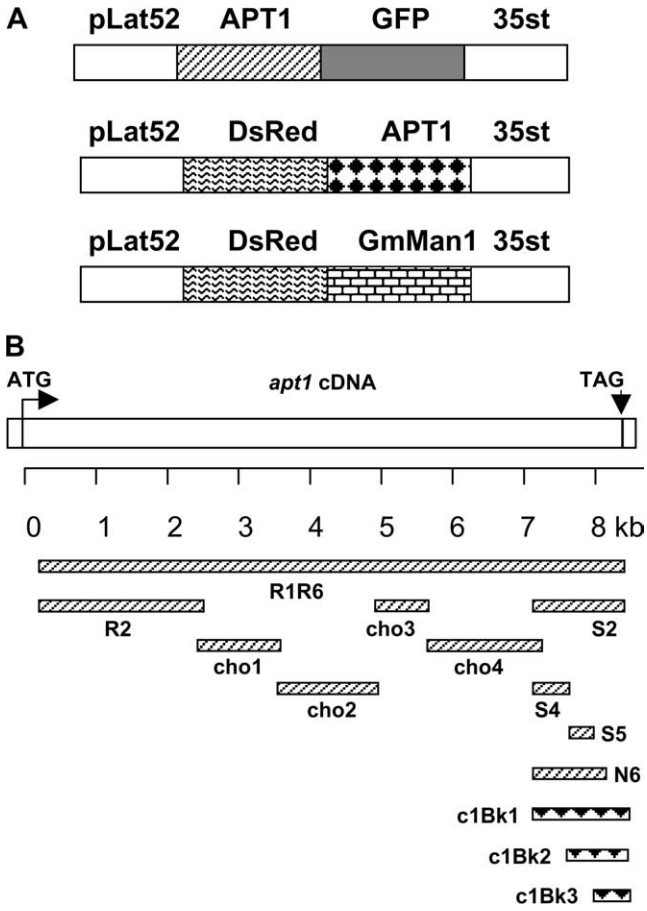

FIGURE 9.—Diagram of constructs used in subcellular localization experiments. (A) General organization of the fusion constructs pLAT52–APT1–GFP (top), pLAT52–DsRed–APT1 (middle), and pLat52–DsRed–GmManI (bottom). pLAT52 is a tomato promoter expressed exclusively in the anthers. 35sT indicates a fragment containing the polyadenylation signal from the cauliflower mosaic virus 35S terminator. (B) Specific APT1–GFP and DsRed–APT1 fusion constructs. The kilobase scale refers to locations in the *apt1* cDNA. APT1–GFP fusions, upward diagonal pattern; DsRed–APT1 fusions, solid triangle pattern.

The distribution of the green fluorescent signal in germinating pollen tubes (Figure 10, A and B) resembled the distribution of proteins, such as Rab2 GTPase, which have been shown to localize to Golgi bodies in elongating pollen tubes (CHEUNG *et al.* 2002). To test whether APT1 also localized to Golgi bodies, a construct was made with the Golgi-targeted GmManI α-1,2 mannosidase I protein (NEBENFUHR *et al.* 1999) fused to DsRed:LAT52–DsRed–GmManI (Figure 9A). Cobombardment of pollen tubes with LAT52–APT1(N6)–GFP and LAT52–DsRed–GmManI showed that APT1(N6) and GmManI are expressed together in the same pollen tube (Figure 10, C and D) and the merged images clearly establish that APT1(N6) and GmManI colocalize to the same organelle (Figure 10E).

The *apt1-m1(Ac)* mutation is a powerful tool for selecting *Ac* transpositions: As reported above, the male

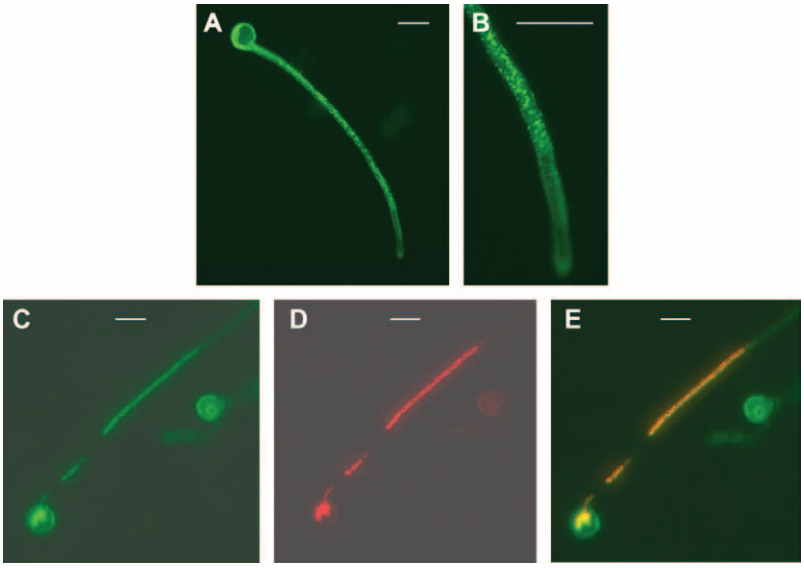

FIGURE 10.—Localization of the APT1(N6)::GFP fusion protein in tobacco pollen tubes. Tobacco pollen was cobombarded with the APT1(N6)::GFP and the DsRed::GmManI constructs. (A) Pollen tube showing a punctate pattern of GFP signals. (B) Enlargement (2×) of the tip of the pollen tube shown in A. (C–E) Images of a different pollen tube. (C) GFP signal; (D) DsRed signal from the same pollen tube as in C; (E) merged images of C and D. Bars, 30 μm.

transmission ratio of *Ac* to wild type in *apt1-m1(Ac)/+* heterozygotes is 1:12. All 16 *Ac* progeny from an *apt1-m1(Ac)/+* pollen parent had a *trAc* element (Figure 4). Therefore, the frequency of *Ac* progeny that carry a *trAc* element is much higher in *apt1-m1(Ac)* than the 1–3% frequency seen in most mutable alleles. The position of the *trAc*s relative to the *apt1-m1(Ac)* donor locus was characterized as follows. First, 57 plants carrying *trAc* elements of independent origin [*bz-m2(D1); trAc/+*] were crossed as males to *bz-m2(D1); apt1-m1(Ac)/+* females. From each of the above crosses, 5 plants derived from bz-mF seeds, which are putative *apt1-m1(Ac)/+* heterozygotes carrying either a linked or an unlinked *trAc*, were pollinated with a *bz-m2(D1)* tester. This testcross generated a segregating population of bz-mF, bz-mC, and bz seed. The genetic distance between the *trAc* and the *apt1-m1(Ac)* donor locus was estimated on the basis of the frequency of bz-mF seeds, which should carry two copies of *Ac* and should arise mostly by recombination between the *trAc* and the *apt1-m1(Ac)* allele. As with other loci, about half of the *trAc*s were found to be linked to the donor locus (supplemental Figure 2 at http://www.genetics.org/supplemental/).

DISCUSSION

The *apt1* mutation is defective in *Ac* male transmission and pollen tube elongation: In this study, we took advantage of two well-known properties of the maize transposon *Ac* to identify and characterize *apt1*, a mutation affecting male gametophytic development. An *Ac trans*-acting phenotype—spotted seed color—was used to follow *Ac* pollen transmission and the negative dosage effect of *Ac* on spot size and number was used to screen for homozygous mutant plants. The *Ac*-linked *apt1* mutation was discovered by the aberrant pollen transmission of *Ac* in *apt1-m1(Ac)/+* heterozygotes. The transmission ratio of *Ac* and non-*Ac* gametes by the pollen is 5:1000, so *Ac* is rarely male transmitted. Heterozygous *apt1* mutant plants show no obvious phenotype other than the defective pollen tube elongation of half of their pollen grains. Rare homozygous *apt1* mutant plants, also apparently normal, were recovered by screening the self progeny of *apt1/+* heterozygotes on the basis of *Ac*'s negative dosage effect and DNA blot genotyping. The *apt1* mutant pollen germinates short and twisted tubes that elongate very slowly. Gametes carrying *Ac* excisions are recovered frequently from *apt1* homozygotes because the revertant pollen tubes elongate much faster than the mutant ones and outcompete them to the embryo sacs.

APT1 is a homolog of the SABRE and KIP proteins required for cell elongation: The *apt1* gene was sequenced from two overlapping BACs from the inbred B73. At 26 kb, *apt1* is one of the longest plant genes known. A full-length *apt1* cDNA was cloned from maize mature pollen. The *apt1* transcript measures 8.6 kb and is made up of 22 exons, which encode a predicted protein of 2607 aa and 292 kDa. *apt1* expression occurs only in mature pollen and depends on the genotype of the gametophyte, making the *apt1* promoter an excellent candidate for a maize pollen-specific promoter.

The APT1 protein is homologous across its entire length to SABRE and KIP, two novel proteins involved in the elongation of root cortex cells and pollen tubes in Arabidopsis. Hereafter, we refer to this plant gene family as SAK (for *S*ABRE, *A*PT1, *K*IP). T-BLASTN analysis of the rice sequences in GenBank using APT1 as query disclosed two high-scoring, but misannotated, homologs in chromosome 3 (AP008209.1). The exon–intron junctions of the rice SAK genes were determined with the Softberry sequence analysis program (http://www.softberry.com/berry.phtml), aided by the maize *apt1* full-length cDNA sequence. The rice homologs are

highly similar to SABRE, APT1, and KIP (supplemental Figure 1): One of them is closer to APT1/KIP and the other one to SABRE. Maize is likely to have at least one other SAK homolog because BLAST searches of the maize Genome Survey Sequence database uncovered related sequences with 80% identity to *apt1*. Thus, monocots and dicots appear to have two closely related SAK family members, but the extent of conservation of function is unclear at this time as, unlike *KIP*, *apt1* is not expressed in roots. APT1 homologs were also detected among pollen or floral RNAs from maize, rice, sorghum, and sugar cane by T-BLASTN translation of the GenBank EST database. Therefore, APT1 is a member of a conserved protein family most likely required for cell elongation in higher plants.

Curiously, different members of the SAK family differ slightly in their intron–exon structure. *SABRE* and *KIP* have 23 exons and completely conserved intron locations relative to each other, whereas *apt1* has 1 exon less. Exon 19 in *apt1* is split into two in SABRE and KIP by an 87-bp intron, which, although defined experimentally, neither removes a stop codon in the spliced sequence nor shifts the reading frame. The two SAK genes in rice appear to have the same genomic organization as *apt1*. A caveat in the preceding discussion is that, except for *apt1*, the complete exon–intron structure has not been determined experimentally for any SAK gene.

APT1 appears to be a Golgi-localized protein required for pollen tube elongation: The pollen tube elongates by rapid apical extension (Franklin-Tong 1999). A highly active inverse fountain cytoplasmic streaming leads to a large accumulation of Golgi-derived vesicles in what is known as the "clear zone" at the extreme tip of the growing pollen tube. There, membrane and cell wall components and secretory proteins are produced to support the pollen tube's rapid elongation (Hepler *et al.* 2001). Recent pharmacological and genetic data point to a major role of the Golgi body in this elongation. The importance of an intact Golgi network for pollen tube elongation was first demonstrated with the organelle movement inhibitor brefeldin A. Treatment of pollen tubes with this drug alters cytoplasmic streaming, causes the disappearance of Golgi bodies, and arrests pollen tube growth (Rutten and Knuiman 1993; Cheung *et al.* 2002; Parton *et al.* 2003). Recent genetic data confirm the importance of the Golgi for pollen tube growth. Mutations in genes encoding Golgi-localized proteins known to regulate vesicle trafficking in animal and yeast cells, such as a tobacco Rab2 GTPase (Cheung *et al.* 2002) and an Arabidopsis homolog of yeast Vps52p (Lobstein *et al.* 2004), are impaired in pollen tube growth. However, the mechanisms by which these proteins regulate pollen tube elongation are not yet understood.

In this study, we have shown that the APT1 protein, which is required for normal pollen tube elongation in maize, may also be targeted to the Golgi body. Different parts of APT1 were fused to GFP or DsRed and bombarded into tobacco pollen. We found that, although the GFP fusion construct containing the entire APT1 was not expressed, possibly because of the unusual length of the APT1 protein, all the constructs containing the carboxy terminus of APT1 produced targeted signals in germinating pollen tubes. A fusion protein of GFP with the APT1 C terminus (residues 2351–2458) colocalized with the Golgi body marker GmManI, indicating that that part of the protein contains a Golgi localization signal. Interestingly, the same part of APT1 shows conservation with animal and yeast proteins of unknown function. We hypothesize that APT1 is a Golgi-localized protein that most likely regulates membrane trafficking and is required for rapid vesicle accumulation at the tip of the pollen tube. Since APT1, KIP, and SABRE are members of a novel protein family and appear to have similar effects on cell elongation, study of the subcellular localization of other SAK proteins could provide clues to their possible mode of action.

The *apt1* mutation constitutes a useful transposition selection tool: We demonstrate here that the *apt1-m1(Ac)* mutation is a very efficient system for selecting *Ac* transpositions in maize. Utilizing the *apt1* mutation as male, a large number of *Ac* transpositions can be recovered in one generation because each ear yields an average of ~10 *trAc*s. Among the transpositions selected, approximately equal numbers are linked and unlinked to the donor *apt1* locus, similar to what has been observed at other loci (Greenblatt 1984; Dooner and Belachew 1989). In the original *apt1-m1(Ac)* mutation, *Ac* is inserted in intron 13. In *apt1-m2(Ac)*, a second *apt1(Ac)* mutant allele isolated recently, *Ac* is inserted in intron 8 (data not shown). This allele shows the same *Ac* male transmission defect as *apt1-m1(Ac)*. Because in these two mutants *Ac* is inserted in an intron, where excision footprints tend to be tolerated, most *Ac* excisions restore gene function and the standard distribution of linked and unlinked *trAc*s is not altered. Therefore, *apt1-m1(Ac)* and *apt1-m2(Ac)* are particularly useful for selecting *Ac* transpositions to closely linked sites. Recovery of an intragenic transposition of *Ac* into an *apt1* exon would increase the versatility of the *apt1* mutation as a paternal donor of *trAc*s because it would provide a tool for selecting *Ac* transpositions to unlinked sites. Most *Ac* excisions from such an allele would not restore gene function and would be subject to the same negative selection as the parental *apt1* mutant allele. Therefore, *Ac*s that transpose to unlinked sites and cosegregate with the wild-type *Apt1* allele would be recovered preferentially, the probability of recovery of a *trAc* being inversely proportional to its linkage to the *apt1* mutation.

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