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KINKY POLLEN AND *POKY POLLEN TUBE* ARE TWO NOVEL GENES REQUIRED FOR TIP GROWTH AND DUPLICATED IN THE *ARABIDOPSIS* GENOME

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Screening of male gametophytic mutants from the Versailles collection of T-DNA transformants allowed us to isolate and characterize two novel genes: *KINKY POLLEN (KIP)* and *POKY POLLEN TUBE (POK)*, which are required for correct tip growth in *Arabidopsis thaliana*. As *KIP* and *POK* are expressed in all plant tissues, though to a higher level in pollen and roots, their roles may not be restricted to tip growth only, but might extend to more general elongation mechanisms. Both genes are duplicated in the *Arabidopsis* genome. Specific roles for each duplicate, indicated by mutant phenotypes, will be discussed. Moreover, KIP and POK proteins have putative orthologs in all eukaryotes investigated, suggesting that they may be crucial proteins required for correct polar growth in all eukaryotic species.

Key words: *Arabidopsis thaliana*, pollen tube, tip growth, duplication.

INTRODUCTION

Due to its central role of the male gametophyte in the plant life cycle, its development has been studied and described well for many years (Bedinger et al., 1994). To gain insight into the molecular mechanisms involved in this process, we and others (e.g., Howden et al., 1998) have undertaken genetic screening of the *Arabidopsis thaliana* T-DNA transformant collection to isolate male gametophytic mutations based on the 1:1 segregation ratio of the kanamycin resistance marker gene carried by T-DNA in selfing progeny (Bonhomme et al., 1998a,b). Two such male gametophytic mutants, *kinky pollen* (*kip*) and *poky pollen tube* (*pok*), both affected in pollen tube growth, have been identified. Pollen tube elongation is a key step of pollen development and fertilization, since it allows the transport of the male gametes to their female counterparts. The pollen tube is a cylindrical structure which elongates following a very efficient and polarized mode of growth, tip growth (Hepler et al., 2001), as a result of apical exocytosis of Golgi vesicles containing parietal precursors. Two intracellular structures are supposed to be particularly important to this process: the cytoskeleton and the Golgi apparatus (Geitmann and Emons, 2000). Tip growth is a mechanism shared by only a limited set of cell types in eukaryotes: pollen tubes and root hairs of plants, fungal hyphae, and animal axons (Palanivelu and Preuss, 2000). Although the molecular players involved in this process are beginning to be highlighted (Hepler et al., 2001), KIP and POK are the first novel proteins described so far that play an important role in the course of tip growth and for which corresponding genes have been cloned. Here we analyze the structure and possible origins and functions of these genes.

MATERIALS AND METHODS

The *Ttd* (*T*-DNA *t*ransmission *d*efect) lines of *A. thaliana* (L.) were isolated from the Versailles collection of T-DNA insertion mutants and cultivated as described in Bonhomme et al. (1998a). Pollen grains from young open flowers were cultured as described by Procissi et al. (2001), and Hodgkin (1983).

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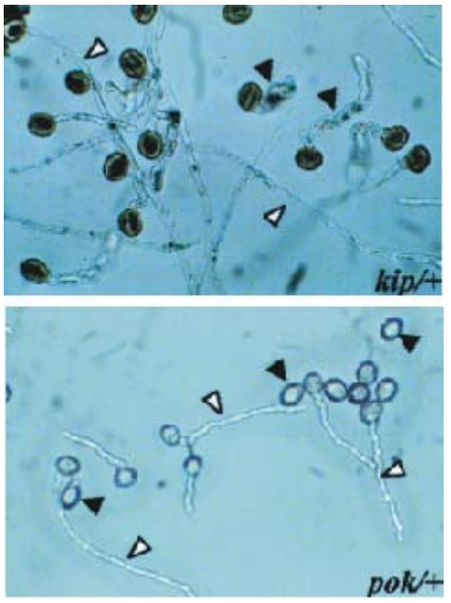


Fig. 1. In vitro germination of pollen grains from hemizygous *kip* (*Ttd26*) and *pok* lines. Arrowheads indicate mutant (black) and WT (white) pollen tube phenotypes.

RESULTS

KIP AND *POK* ARE TWO MALE GAMETOPHYTIC MUTATIONS AFFECTING TIP GROWTH

Transmission defects through the pollen for three allelic *kip* mutant lines (*Ttd26, Ttd34* and *Ttd42*) and the single *pok* mutant line (*Ttd8*) have been described in Bonhomme et al. (1998a) and Procissi et al. (2001).

In vitro pollen germination experiments (Fig. 1) show that both mutations affect pollen tube elongation. For *kip* hemizygous mutants, 50% of the pollen tubes are twisted and sometimes branched. The kinky shape of *kip* pollen tubes results from growth arrests and reorientations of the growth axis, as shown by Procissi et al. (submitted). The penetrance of the mutation differs between the three *kip* alleles, *Ttd34* being a null one. Shorter and thicker root hairs are also observed in *kip* homozygous lines (not shown), confirming the involvement of the *KIP* gene in tip growth.

When cultivated in vitro, pollen from the hemizygous *pok* mutant have 50% of the pollen tubes shortened, even after 12 h culture. This and previously published T-DNA transmission data (Bonhomme et al., 1998b) suggest that tip growth either is precociously arrested or is severely slowed. To investigate the potential root hair mutant phenotype, *pok* homozygous mutants were searched, without success, suggesting that the homozygous *pok* mutation leads to embryo lethality.

KIP AND *POK* ARE EXPRESSED IN ALL PLANT TISSUES

Study of the expression patterns of both genes (not shown) indicates that *KIP* and *POK* transcripts are expressed in all plant tissues at a low level; however, expression levels were higher in roots and anthers for the *KIP* gene, and in roots and flower buds for the *POK* gene.

BOTH *KIP* AND *POK* SEEM TO BE DUPLICATED IN THE *ARABIDOPSIS* GENOME

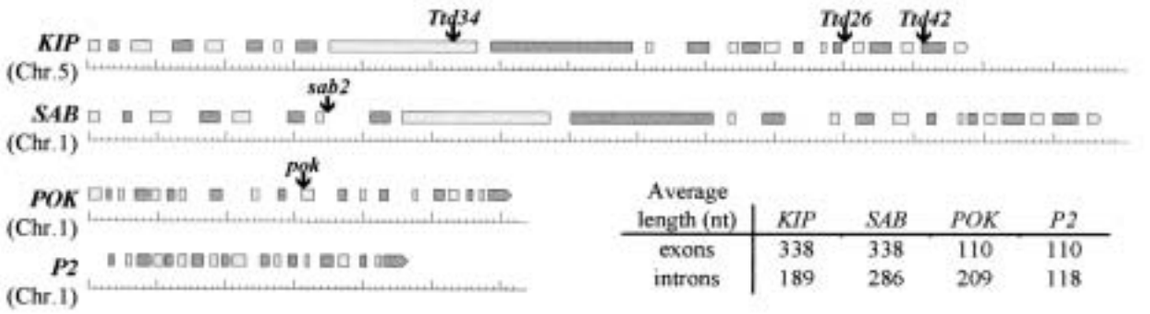
The *KIP* gene is located on chromosome V and transcribed in a 7.8 kb long fragment of mRNA exhibiting 66% identity with *SABRE* (*SAB*) cDNA. The *SAB* gene is located on chromosome I, and *sab1* mutants have been described (Aeschbacher et al., 1995). While homozygous *sab* mutants show a global dwarf phenotype, no abnormal phenotype has been observed in tip-growing cells (pollen tubes or root hairs). The *SAB* gene is expressed in all tissues at a constant level, more or less similar to the basal level of *KIP* transcript expression (not shown).

The *POK* gene is located on chromosome I and is transcribed in a 2.2 kb mRNA fragment. Four kb downstream from the *POK* gene is another gene which we named *P2*, whose transcript exhibits 90% identity with the *POK* transcript. No *P2* mutant is available so far, but RT-PCR experiments suggest that the *P2* gene is expressed in all plant tissues, although at a very low level.

For both *KIP*/*SAB* and *POK*/*P2* pairs, the intron/exon structures are highly conserved between duplicated genes (Fig. 2), with a strong identity between exon sequences, whereas intron sequences are totally divergent. *KIP* and *SAB* both contain 23 exons, sharing 44% to 80% identity. The *POK* and *P2* exons (20 and 19, respectively; *POK* having an additional 5' exon) share 79% to 98% identity. On average, *SAB* introns are longer than *KIP* introns, whereas introns are shorter in *P2* than in *POK*.

DEDUCED KIP AND POK PROTEINS ARE CONSERVED AMONG EUKARYOTES

Deduced KIP and POK proteins (2587 and 708 amino acids, respectively) have putative orthologs in many eukaryotic species, from yeast to human (Tab. 1). In each case, homologies are spread over the entire protein sequence. The function of the KIP



Average length (nt)	*KIP*	*SAB*	*POK*	*P2*
exons	338	338	110	110
introns	189	286	209	118

Fig. 2. Structure of *KIP*, *SAB*, *POK* and *P2* genes. Even exons are colored light grey, uneven in dark grey, to facilitate comparison of duplicated gene structures. Arrows indicate T-DNA insertion positions for the different mutants available. *sab2* is a mutant from the Versailles *Arabidopsis* T-DNA transformant collection (our unpublished data).

protein is still unknown, but prediction software packages identify a signal peptide that could target it to the secretory pathway. The *Saccharomyces cerevisiae* putative ortholog of POK, Vps52p, has been shown to be involved in the recycling machinery of Golgi-resident proteins (Conibear and Stevens, 2000).

DISCUSSION

kip and *pok* are two male gametophytic mutations affecting tip growth. The sequences of KIP and POK proteins are conserved among numerous plant and animal species in which tip-growing cells have been described. This high conservation suggests a crucial function for both proteins. Both the location and function of the KIP protein remain to be elucidated, although a role in extracellular matrix organization can be hypothesized (unpublished results).

The role of its putative yeast ortholog, Vps52p, in Golgi vesicle recycling, suggests that the function of POK could extend to more general growth mechanisms and not be restricted to tip growth only. This idea is reinforced by different observations: (i) the *POK* gene is ubiquitously expressed, (ii) the POK protein is abundantly present in the root elongation zone (not shown), and (iii) the *pok* homozygous mutation is suspected to lead to embryo lethality. Thus, POK could be required for correct Golgi vesicle trafficking in any elongating or dividing cell.

Duplication of both the KIP and POK genes is not very surprising, since it is now thought that *Arabidopsis thaliana* must be a degenerate tetraploid, following a duplication of its whole genome 112 million years ago (Ku et al., 2000). As is the case for the POK and P2 genes, a large number (17%) of *Arabidopsis* genes are tandemly repeated, whereas other portions of the genome have been subsequently rearranged, leading to the separation of the duplicated genes, as for *KIP* and *SAB* (The Arabidopsis Genome Initiative, 2000; Blanc et al., 2000).

Genome evolution studies demonstrate that a gene duplicate can follow four fates (Lynch and Conery, 2000) for which examples are known in plant genomes: (i) true and complete redundancy is conserved between both duplicates (e.g., *SHP1* and 2 genes of *Arabidopsis*) (Liljegren et al., 2000); (ii) nonfunctionalization: one copy is silenced by degenerative mutations, while the other maintains its initial function (e.g., *TGG3* of *Arabidopsis*) (Zhang

TABLE 1. Protein sequence comparisons between KIP, POK and their putative orthologs in few eukaryotic species

Species	KIP			POK		
	%identity	%similarity	Acc.number	%identity	%similarity	Acc.number
Arabidopsis thaliana:SAB	57	76	AAC49734	–	–	–
A. thaliana:P2	–	–	–	88	93	AAG51887
Saccharomyces cerevisiae	12	34	AAB68087	23	43	AAB64912[a]
Caenorhabditis elegans	13	32	CAB07193	26	47	AAA68727
Drosophila melanogaster	13	34	AAF47740	32	55	AAF52254
Homo sapiens	13	32	BAA07891	34	57	AAH32108

[a] corresponding to Vps52p

et al., 2000); (iii) neofunctionalization: one copy is conserved in its original fate while the second evolves to assume a new beneficial function and is thus preserved (e.g., chalcone synthase shift of function into stilbene synthase in *Antirrhinum*) (Durbin et al., 2000); and (iv) subfunctionalization: both copies are preserved and both of them fix complementary loss-of-function mutations (e.g., *WER* and *GL1* genes of *Arabidopsis*) (Kellogg, 2001).

Considering both *KIP*/*SAB* and *POK*/*P2* pairs, redundancy can clearly be dismissed, as the mutant phenotype can be observed when one gene of the pair is mutated. *KIP* and *SAB* genes might correspond to the neofunctionalization case, as it seems that one gene (*SAB*) is involved in diffuse growth processes, whereas the other one (*KIP*) became specialized for tip-growing cells. This is suggested by the *kip* mutant phenotype and the *KIP* expression pattern. Concerning the *POK*/*P2* duplication, the possibilities of neofunctionalization, subfunctionalization and nonfunctionalization (i.e., considering *P2* as evolving to a pseudogene) can also be hypothesized. There are several examples of "young pseudogenes" that have conserved their exon/intron structure and are still expressed, though at a very low level (Ramos-Onsins and Aguadé, 1998; Zhang et al., 2000). We are now searching *P2* mutant lines to discriminate between these hypotheses.

It is interesting that characterization of two independent male gametophytic mutations led us to two novel genes highly conserved among eukaryotes, both of which are involved in polarized growth and duplicated in *Arabidopsis*. Further study of both KIP and POK proteins should provide new insights about tip growth and more general elongation processes, and perhaps yield new clues to understanding the wide redundancy of the *Arabidopsis* genome.

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