The Putative Arabidopsis Homolog of Yeast Vps52p Is Required for Pollen Tube Elongation, Localizes to Golgi, and Might Be Involved in Vesicle Trafficking

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The screening of the Versailles collection of Arabidopsis T-DNA transformants allowed us to identify several male gametophytic mutants, including poky pollen tube (pok). The pok mutant, which could only be isolated as a hemizygous line, exhibits very short pollen tubes, explaining the male-specific transmission defect observed in this line. We show that the POK gene is duplicated in the Arabidopsis genome and that the predicted POK protein sequence is highly conserved from lower to higher eukaryotes. The putative POK homolog in yeast (Saccharomyces cerevisiae), referred to as Vps52p/SAC2, has been shown to be located at the late Golgi and to function in a complex with other proteins, Vps53p, Vps54p, and Vps51p. This complex is involved in retrograde trafficking of vesicles between the early endosomal compartment and the trans-Golgi network. We present the expression patterns of the POK gene and its duplicate P2 in Arabidopsis, and of the putative Arabidopsis homologs of VPS53 and VPS54 of yeast. We show that a POK::GFP fusion protein localizes to Golgi in plant cells, supporting the possibility of a conserved function for Vps52p and POK proteins. These results, together with the expression pattern of the POK::GUS fusion and the lack of plants homozygous for the pok mutation, suggest a more general role for POK in polar growth beyond the pollen tube elongation process.

Pollen tube growth is a vital process during male gametophyte development, since it allows male gametes to reach the ovules and achieve fertilization (Preuss, 1995). The elongation of the pollen tube, as well as that of animal axons, plant root hairs, fungal hyphae, and moss protonema, is achieved by a polarized mode of growth, termed tip growth (Franklin-Tong, 1999; Palanivelu and Preuss, 2000; Hepler et al., 2001), which involves the tip-localized exocytosis of Golgi-derived vesicles containing cell wall precursors (Franklin-Tong, 1999). The tip of the pollen tube is devoid of organelles, but contains almost exclusively Golgi-derived vesicles (Geitmann and Emons, 2000). In the shank of the tubes, an inverse fountain pattern of cytoplasmic streaming is observed; organelles and vesicles are transported toward the apex in the cortical and basipetal region in the central cytoplasm (Pierson et al., 1990). Actin filaments and microtubules, which are organized in longitudinal arrays more or less parallel to the elongation axis, act as tracks for cytoplasmic streaming and allow delivery of vesicles to the tip (Pierson et al., 1990; Vidali and Hepler, 2001). The use of membrane structure dyes, such as FM1-43 or FM4-64 (Parton et al., 2001; Camacho and Malhó, 2003), has revealed dynamic endo/exocytosis processes at the tip of the pollen tube, but the molecular events underlying these processes are still poorly understood. The recent identification of the Golgi-associated tobacco (Nicotiana tabaccum), Rab GTPase NrRab2, predominantly expressed in pollen, suggests that tip growth and vesicle trafficking could be tightly linked (Cheung et al., 2002). Moreover, a GTP-bound dominant negative form of NrRab2 blocks endoplasmic reticulum to Golgi trafficking and inhibits pollen tube growth (Cheung et al., 2002).

The functions of numerous vesicle-trafficking proteins and several different protein-sorting pathways are well described in yeast (Saccharomyces cerevisiae; for review, see Kucharczyk and Rytkä, 2001). Although many homologs of these proteins have been identified in plants (e.g. Ahmed et al., 1997; Conceição et al., 1997; Bassham and Raikhel, 1998; Sanderfoot et al., 1998; Zheng et al., 1999), little is known about their precise roles. However, knowledge from yeast has accelerated progress in identifying plant vacuolar protein complexes. For example, the isolation of an Arabidopsis insertion mutant for the VACUOLELESS 1 gene (Rojo et al., 2001), which encodes a protein homolog of the yeast Vps16p, recently lead, through comparison with the yeast model, to the characterization of three components of the first vacuolar complex.

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identified in plants (Rojo et al., 2003). Considering the differences in cellular organization and the higher number of potential secretory pathway proteins in plant versus yeast (Sanderfoot et al., 2000; Jürgens and Geldner, 2002), it seems likely that the vesicle-trafficking machinery has undergone specific modifications in plants (Bassham and Raikhel, 2000; Jürgens and Geldner, 2002).

Using insertional mutagenesis, we identified several putative male gametophytic mutants (Bonhomme et al., 1998a, 1998b; Procissi et al., 2001), which included four affecting the specific step of pollen tube development. The poky pollen tube (pok) mutant, previously named A9 or Ttd8, exhibits very reduced pollen tubes when grown in vitro (Bonhomme et al., 1998b). In this article, we show that the POKY POLLEN TUBE (POK) gene is duplicated in the Arabidopsis genome and is ubiquitously expressed in plant tissues. However, the expression pattern of a POK::GUS fusion protein suggests higher protein expression in roots and reproductive tissues. The predicted POK protein shares significant homology with the yeast Vps52p/SAC2 protein, which has been shown to be involved in the retrograde transport of Golgi-derived vesicles toward the late Golgi (Conibear and Stevens, 2000). We report the cloning of Arabidopsis genes encoding putative homologs of the Vps52p protein partners and comparison of their expression patterns with that of the POK gene. Furthermore, a POK::GFP fusion protein localizes to Golgi structures in living plant cells, supporting the hypothesis of conservation of the function of both POK and Vps52p proteins in Arabidopsis.

RESULTS

The pok Phenotype Affects Pollen Tube Tip Growth

In order to define the pok phenotype in situ, aniline blue staining was performed after limited pollination of wild-type pistils with pollen grains, either from wild-type or from hemizygous pok plants. Sixteen hours after pollination, all wild-type pollen grains had germinated and completely elongated their pollen tubes, reaching the base of the ovary (Fig. 1a). The number of pollen tubes counted in the style was similar to the number of pollen grains deposited (Fig. 1a, n = 7). On the contrary, pollen from pok plants produced both elongated and short pollen tubes (Fig. 1b, five pollen grains on stigma, three tubes in the ovary). When only one or two grains were deposited, we commonly observed short, poky pollen tubes 16 h after pollination, which are presumed to carry the T-DNA construct (Fig. 1c). This observation is consistent with a role for the POK gene in pollen tube growth in vivo.

Nonetheless, some pollen tubes were still able to elongate sufficiently to reach ovules located in the upper part of the ovary (Fig. 1c). This would explain the residual male gametophytic T-DNA transmission (0.46%) in the pok mutant line (Bonhomme et al., 1998a, 1998b). Despite this residual transmission, numerous screens to isolate homozygous pok plants remained unsuccessful, suggesting that homozygous pok mutations lead to precocious lethality.

POK Is Duplicated in the Arabidopsis Genome, but the pok Phenotype Can Be Assigned to the POK Gene Alone

Genomic regions flanking the single T-DNA construct inserted in the pok mutant line were cloned (Bonhomme et al., 1998a) and sequenced, leading to the identification of a pair of duplicated genes located 4 kb apart on the bottom of chromosome I (Fig. 2a): the POK gene, in which the T-DNA insertion occurred (At1g71270), and the P2 gene (At1g71300).
The POK and P2 genes (Fig. 2a) are composed of 20 and 19 exons, respectively (with an additional 5' exon in POK). The exon-intron structure (Fig. 2a) is strictly conserved and exon sequences share 90% identity, whereas intron sequences are largely divergent and shorter in P2 than in POK.

Pok hemizygous mutant plants were complemented with an 11.2-kb genomic clone overlapping the POK gene, but excluding P2. The complementation construct was linked to the hpt gene, whereas the pok T-DNA insertion was marked with the nptII gene, conferring hygromycin (H) and kanamycin (K) resistance, respectively, to the transformed plants. Among the progeny of infiltrated mutant plants, five plants were selected that, when selfed, showed 2:1 segregation for kanamycin resistance, instead of 1:1 observed in the pok mutant. Furthermore, these five plants showed 5:1 segregation for hygromycin resistance, as expected in the case of independent segregation of both T-DNA insertions. The self progeny of one of these potentially complemented plants was analyzed. Descendants homozygous for hygromycin resistance either were also homozygous for kanamycin resistance (H/H, K/K), or showed a 3:1 ratio of kanamycin-resistant to kanamycin-sensitive progeny (H/H, K/-). Crossing these latter plants as male in with wild type confirmed that T-DNA transmission was restored. Moreover, descendants that had lost the complementing construct (showing a fully hygromycin-sensitive progeny) showed 1:1 segregation for kanamycin resistance. Finally, when germinating pollen from a complemented descendant (K/-, H/H) in vitro, the same proportion of elongated pollen tubes (78%, out of 348 counted pollen grains) was obtained as for wild-type pollen (77% of elongated tubes, out of 340 pollen grains), showing phenotypic restoration for this plant.

To rule out potential lesions in the linked P2 gene, the P2 genomic region was completely sequenced in the pok mutant and no mutations were observed. Taken together, these data confirm that the T-DNA insertion in the POK gene alone is responsible for the observed tip growth defect in pok plants.

Expression of the POK and P2 Genes

Northern-blotting experiments using exon 10 of the POK gene as a probe (Fig. 2b) revealed a transcript of the expected size (2.2 kb) at every developmental stage tested and in almost all plant tissues. However, there was some variation in transcript abundance; very low levels of transcripts were detected in seeds, whole inflorescence, and mature flowers, while higher levels were observed in roots and flower buds less than 1 mm in size. Due to their strong identity, the POK and P2 transcripts could not be distinguished in hybridization experiments. However, oligonucleotides specific for each cDNA were designed (Fig. 2, c and d) and reverse
transcription (RT)-PCR analysis was used to characterize the expression pattern of both transcripts independently. For the POK gene, RT-PCR results, although not quantitative, were qualitatively similar to those obtained by northern blotting, with the POK cDNA detected in all tissues, although at very low levels in seeds (Fig. 2e). For the P2 cDNA, no RT-PCR products were detected in any sample (Fig. 2f), even after two rounds of nested amplification (not shown).

Taken together, the weak, or absent, expression of P2 versus POK and the presence of the POK cDNA in all RT-PCR samples argue that the northern-blot analysis shown in Figure 2b faithfully reflects POK mRNA distribution only.

The POK and P2 Transcripts

Pollen and root cDNA libraries were screened with a probe corresponding to the T-DNA insertion region and overlapping the predicted exon 10 of the POK gene. Two types of clones were obtained from the pollen cDNA library: a POK partial cDNA clone (1,793 nt long) and a P2 cDNA clone (2,096 nt long). The screening of the root cDNA library allowed the isolation of a longer POK cDNA clone (2,217 nt); however, no cDNA clone for the P2 gene was found.

Putative initiator ATGs for both transcripts (NetStart prediction software: http://www.cbs.dtu.dk/services/NetStart/), located in exon 1 (Fig. 2a), predict a total length for POK and P2 coding sequences of 2,088 and 2,106 nt, respectively. 5’RACE experiments using POK-specific primers indicated a 5’UTR of 156 to 158 nt, located 31 to 33 nt upstream of the 5’ terminus of the root cDNA.

POK and P2 Are Highly Conserved among Eukaryotes

The POK and P2 predicted proteins are 696 and 702 amino acids, respectively, with a predicted molecular mass of approximately 80 kD. Both proteins share 88% identity and 93% similarity. According to BLAST software, POK shows 23% identity and 43% similarity with the yeast SAC2/Vps52p protein (Kölling et al., 1994; Conibear and Stevens, 2000). Predicted homologous proteins are also found in other species, such as Homo sapiens, Drosophila melanogaster, and Caenorhabditis elegans, with 23% to 35% identity and 43% to 59% similarity over the entire sequences (Fig. 3). Although some amino acids appear well conserved among the different species (Fig. 3), no particular structure, signal peptide, or known motifs are predicted in the POK sequence.

The AtVPS53 and AtVPS54 Genes

Conibear and Stevens (2000) showed that the yeast Vps52p protein functions in complex with two other proteins called Vps53p and Vps54p. We therefore searched for Arabidopsis genes encoding potential homologs of Vps53p and Vps54p. Performing BLAST analysis, we identified two candidate genes that we called AtVps53 and AtVps54 (At1g50500 and

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Figure 3. POK is conserved among eukaryotes. Alignment of POK and P2 protein sequences with their putative orthologs in S. cerevisiae (Vps52p, accession, AA64912), H. sapiens (H.s, accession, AAH32108), D. melanogaster (D.m, accession, AAF52254), and C. elegans (C.e, accession, AAA68727), according to ClustalW software (http://npsa-pbil.ibcp.fr/cgi-bin/npsa_automat.pl?page=NPSA/npsa_clustalw.html). Black boxes, Amino acids identical to POK amino acids. Gray boxes, Amino acids strongly similar to POK amino acids.
At4g19490, respectively). The corresponding predicted proteins share, respectively, 19% and 22% identity and 38% and 44% similarity with their yeast counterparts.

AtVps53 and AtVps54 cDNA clones were isolated from the pollen cDNA library. They are both 2.7 kb long and contain 24 and 18 exons, respectively (Fig. 4, a and d). The predicted proteins contain 829 and 1,001 amino acids, respectively, compared to 822 and 889 amino acids for Vps53p and Vps54p. A predicted translation start for the AtVps53 transcript is located in the middle of exon 2 (Fig. 4a, black arrow). For AtVps54, GENSCAN software (Chris Burge, Stanford University) predicts a long exon 1 (Fig. 4d, gray arrow), but RT-PCR experiments did not allow amplification of this region (not shown). The first in frame ATG of the isolated AtVps54 pollen cDNA is located at the end of exon 1 (Fig. 4d, black arrow), which would give rise to a 784-amino acid protein.

Expression of these two vps-like genes was studied by northern-blot analysis (Fig. 4, b, c, e, and f). As observed for POK, both AtVps53 and AtVps54 are ubiquitously expressed. Their basal expression level is low, although higher for AtVps53 compared to AtVps54 in all vegetative tissues (Fig. 4, b and e), and higher for AtVps54 in roots versus other tissues (Fig. 4e). In reproductive tissues, both transcripts are more abundant in buds less than 1 mm in size compared with later stages (Fig. 4, c and f).

Distribution of the POK::GUS Fusion Protein

Since an active translational fusion between the first 10 exons of the POK gene and the uidA gene carried by the T-DNA occurred in the pok mutant, GUS staining allowed us to determine a preliminary expression pattern for the POK protein. In 8- to 10-d-old plantlets, strong blue staining was detected in the root apex (Fig. 5a), with enhanced intensity in the elongation zone (Fig. 5b), in emerging lateral root primordia (Fig. 5c), in very young leaves (Fig. 5d), and sometimes in stipules (Fig. 5a).

In reproductive tissues, GUS staining was observed at different stages of floral development (Fig. 5, e–j). Blue coloration was detected from the earliest stages of flower development, before meiosis and gametogenesis (bud size, 0.1 mm; Fig. 5e), and maintained later (Fig. 5, f–j). As flower bud size reached 1.6 to 1.7 mm (i.e. after PMII), expression of the fusion protein became strictly gametophytic in the male reproductive tissues, whereas it was clearly sporophytic in the female tissues, since all ovules were stained (Fig. 5h). At maturity, half of the pollen grains within the anthers were blue (Fig. 5i), as expected for hemizygous plants. At later postpollination stages, all developing seeds were stained (Fig. 5i). However, no blue staining was observed in the pistil below the stigma surface (Fig. 5i), compared to a control line (T-DNA insertion line; A. Guyon, A. Lécureuil, P. Guerche, and S. Bonhomme, unpublished data) expressing GUS in pollen and for which a blue coloration was observed in the style, corresponding to elongated pollen tubes (Fig. 5k; note that developing seeds are not stained). The absence of staining in the style of the pok mutant could be the result of lower GUS expression in this line compared to our control line, as well as of lack of effective pok pollen tube growth.

POK Is Localized to Golgi in Living Plant Cells

The subcellular localization of the POK protein was investigated using a POK::GFP fusion construct
Figure 5. GUS staining assays on hemizygous pok mutants. GUS staining on 10-d-old plantlets (a and d), 8-d-old roots (b and c), and successive stages of floral development (e–j). e, premeiotic stage, pistil size, 0.1 mm; f, tetrad stage, pistil size, 0.5 mm; g, microspore/PMI stage, pistil size, 0.8 mm; h, trinucleate pollen stage, pistil size, 2 mm; i, postpollination stage; j, trinucleate pollen stage; k, control transgenic (hemizygous) line, expressing GUS in pollen grains and pollen tubes, postpollination stage.
containing the complete open reading frame of the 
Poku gene fused with the GFP coding sequence. Both 
cDNAs were cloned downstream of 2.2 kb of the pre-
predicted Poku promoter region. Onion (Allium sativum) 
epidermal cells were bombarded with this Poku::GFP 
construct. The collected signal exhibited a cytoplasmic 
dispersed punctate pattern (Fig. 6a) characteristic of 
the previously described Golgi distribution pattern in 
plant cells (Boevink et al., 1998; Batoko et al., 2000). 
Onion cells were also cobombarded with both the 
Poku::GFP construct and a ST::DsRed construct 
(provided by F. Brandizzi, Oxford Brookes University, 
Oxford, UK; Saint-Jore et al., 2002), where the signal 
anchor sequence of the rat α-2,6-sialyltransferase 
gene was used as a late Golgi marker. Both GFP (Fig. 
6c) and DsRed (Fig. 6b) signals exhibited a very similar 
distribution within the cell. The overlay and coinci-
dence of both signals (Fig. 6d) strongly suggests that 
the Poku::GFP fusion protein localizes to Golgi in 
onion epidermal cells.

DISCUSSION

Poku and Pollen Tube Elongation: Slowly to the Goal

pok can be classed a late gametophytic mutant, since 
microsporogenesis and gametogenesis show no phe-
notypic differences compared to wild type. Moreover, 
we showed that pollen tube elongation is impaired in 
pok. Contrary to the raring-to-go Arabidopsis mutant 
(Johnson and McCormick, 2001), initial steps of pollen 
germination do not seem to be affected in pok. Indeed, 
in vitro germination (Bonhomme et al., 1998b) and in 
situ pollination experiments both show that at least 
some pok pollen grains are able to germinate, but 
pollen tubes subsequently fail to elongate. The limited 
transmission of the pok T-DNA insertion after pollin-
nating wild-type pistils with pollen from the pok 
mutant suggests that, although reduced, elongation 
is still possible in pok pollen tubes. We previously 
showed (Bonhomme et al., 1998b) that the percentage 
of male transmission obtained after such crosses is 10 
times higher in the upper third of the ovary (i.e. the 
ovary fraction located beneath the style). This obser-
vation leads to the hypothesis that the T-DNA trans-
mission defect observed in pok line selfing progeny 
likely results from a competition between the pok 
pollen tubes and the wild-type pollen tubes. We also 
conclude that the pok mutation strictly affects the tip 
growth processes in pollen, since pok pollen tubes that 
are able to reach the upper ovules subsequently 
accomplish fertilization.

POK Function May Not Be Restricted to Tip Growth

Although weak T-DNA transmission through the 
male gametophyte has been observed, suggesting 
incomplete penetrance of the mutation, no plants 
homozygous for the pok mutation were isolated. This 
suggests that the POK function is not restricted to

Figure 6. Localization of the POK::GFP fusion protein in onion epidermal cells. Onion epidermal cells were cobombarded with 
the POK::GFP and the ST-DsRed constructs. Resulting fluorescent signals have been collected independently. a and c, GFP 
signal; b, DsRed signal; d, overlay of b and c. Scale bar represents 80 μm.
reproduction, but could also be essential in early developmental events. This hypothesis is reinforced by the expression pattern of the POK gene, which is clearly not restricted to the reproductive organs, but extends to other plant tissues. Indeed, northern-blot experiments showed that, apart from reproductive tissues (see below), POK expression is high in elongating tissues, such as roots or young seedlings. Moreover, intense GUS staining is detected in the elongation zone of the root apex and in the emerging lateral roots of the pok mutant.

POK Expression in Reproductive Tissues

Even though the POK gene basal expression level is quite low, we detected higher levels of POK transcript in young flower buds (i.e. before the uninucleate microspore stage) compared with later stages of floral development. POK gene expression thus seems to be restricted to early steps of microgametogenesis, allowing us to classify it with the early class of pollen-expressed genes, which are transcribed soon after meiosis and whose transcripts accumulate until PMII and then decrease (Mascarenhas, 1990). A similar expression pattern has been reported for the GRP2 mRNA of tobacco (Ylstra and McCormick, 1999) and for some pollen-expressed Petunia genes (Guyon et al., 2000). In addition, GUS assays on pok inflorescences revealed precocious blue staining, which was maintained throughout flower development. In male reproductive organs, the staining appears strictly gametophytic, from PMII until maturity. Nonetheless, the mutant phenotype suggests that the POK protein is only required for the later steps of male gametophyte development (i.e. correct elongation of the pollen tubes). As the POK transcript seems to be unstable in the developing pollen grain, one can hypothesize that it would be rapidly translated and that the POK protein, rather than the POK mRNA, would accumulate in maturing pollen grains. It has been clearly shown that 5'-UTR are involved in both transcriptional and translational regulations of many plant genes (Bate et al., 1996; Jun Hua et al., 2001; Hulzink et al., 2002); investigation of the precise role of the POK gene 5'-UTR may provide new insights into the complex regulation of the early class of pollen-expressed genes.

The transcript level of putative homologs for VPS53 and VPS54 in Arabidopsis, named AtVps53 and AtVps54, was studied. Expression levels of both genes were revealed to be quite weak (particularly for AtVps54); however, expression was higher in roots (mainly for AtVps54). For both AtVps53 and AtVps54, an expression pattern comparable to that of POK was observed in reproductive tissues (i.e. higher expression in young flower buds), suggesting that they also could be part of the early class of pollen-expressed genes. It has been shown that, for a given gene, mRNA stability can be different in pollen and in somatic cells (Ylstra and McCormick, 1999). One can thus hypothesize that, at least in male gametophytes, POK, AtVps53, and AtVps54 transcripts would share the same type of regulation, involving rapid translation of unstable transcripts and subsequent protein accumulation.

POK and P2

A second mRNA, sharing 90% identity with POK and named P2, is also expressed in pollen grains, although at a much lower level, as it was undetectable using RT-PCR. Similar to 17% of the full complement of Arabidopsis genes (Blanc et al., 2000), POK and P2 are two tandemly repeated duplicated genes. We can hypothesize that P2 could be a POK-derived pseudogene, although Blanc et al. (2000) suggest that a highly conserved exon-intron structure between duplicated genes is an indication that both genes are expressed. However, examples of young pseudogenes that have conserved their exon-intron structure and are still expressed at very low levels have been described in Drosophila (Ramos-Onsins and Aguade, 1998), mouse (Hirotsune et al., 2003), and Arabidopsis (Zhang et al., 2000). Identification and phenotypic analysis of P2 mutant lines should help us to establish the role of the P2 gene. It is important to note that, despite the high identity between both transcripts and both predicted proteins, POK and P2 genes are not fully functionally redundant, at least in the male gametophyte where they are both expressed.

POK Shows Significant Homology with Vps52p

The deduced protein sequences of both POK and P2 share 23% to 35% identity with several eukaryotic proteins, all of which seem to be orthologous to one another, including the SAC2/Vps52p protein of yeast. The VPS52 gene, as well as VPS53 and VPS54, were identified by Conibear and Stevens (2000) while screening for novel components of the trans-Golgi network-localized vesicle-transport machinery. Vps52 was found to be allelic to the previously identified SAC2 gene (Novick et al., 1989; Kölling et al., 1994). It was shown that Vps52p, Vps53p, and Vps54p form a stable complex that is peripherally associated with the late Golgi compartment and is involved in the recycling of resident Golgi proteins from an early endosomal, or prevacuolar, compartment to the trans-Golgi network (Conibear and Stevens, 2000). This hypothesis was reinforced when Siniossoglou and Pelham (2001) showed that Vps52p could bind both the Ypt/Rab GTPase Rab6 and the t-SNARE Tlg1p. More recently, a fourth subunit of the VPS complex with a potential regulatory function has been identified in yeast and was named Vps51p (Conibear et al., 2003; Reggiori et al., 2003).

Proteins shown to be components of the vesicle-transport machinery are usually well conserved among eukaryotic species from yeast to human. As observed for POK, most of these genes have been
shown to be ubiquitously expressed, but to higher levels in roots (Ahmed et al., 1997; Conceição et al., 1997; Bassham and Raikhel, 1998; Rojo et al., 2003). Although many genes homologous to those involved in the yeast Golgi vesicle trafficking have already been identified in Arabidopsis (e.g. Ahmed et al., 1997; Conceição et al., 1997; Bassham and Raikhel, 1998; Sanderfoot et al., 1998; Zheng et al., 1999), to our knowledge POK would be one of the first for which a corresponding mutant line has been identified.

POK Could Be Involved in Plant Vesicle Trafficking

Using POK::GFP and ST::DsRed fusion constructs in transient assays, we demonstrated that POK shares the same localization in plants as Vps52p does in yeast. Since tip-growing cells, such as pollen tubes, ensure rapid and polarized elongation through active apical exocytosis of Golgi vesicles (Hepler et al., 2001), the phenotype observed in pok mutants can be explained if the POK protein has a function comparable to that of Vps52p. Indeed, if POK is involved in the recycling of Golgi resident membrane proteins, alteration of its function would lead to inefficient vesicle recycling and subsequent decrease of the growth rate. Furthermore, it seems that POK could also have a more general role in non-tip-growing cells, where vesicle-recycling deficiency could also lead to slow growth, explaining the proposed zygotic lethality of homozygous pok mutants. The study of the pok mutant should allow us to progress in the understanding of the molecular mechanisms involved in pollen development and pollen tube growth. Since POK seems to belong to the early class of pollen genes and exhibits a complex expression pattern, study of its regulation could be of particular interest. Besides, the further characterization and analyses of the functions of POK should provide new insights concerning the processes governing not only tip growth, but also vesicle trafficking in the male gametophyte and in somatic cells.

Complementation Experiments

A Xbal/Sall 11.2-kb fragment corresponding to the POK gene was subcloned from a genomic phage clone (Bonhomme et al., 1998a) and inserted into a binary vector (pZP P20; Hajdukiewicz et al., 1994) bearing the hpt gene. The hemizygous pok line was transformed by floral dipping in an Agrobacterium solution as described by Clough and Bent (1998). Transformants were selected in vitro on hygromycin-containing medium (30 mg/L).

Screening of cDNA Libraries

The pollen cDNA library was constructed from poly(A+) RNA isolated from mature pollen of Arabidopsis ecotype Landsberg erecta. Mature pollen was isolated as described previously (Hensy and Twell, 2003). Poly(A+) RNA was isolated directly from isolated pollen after grinding pollen in liquid nitrogen using a pestle and mortar and using the Dynabeads mRNA DIRECT mRNA kit (Dynal Biotech, Oslo) according to the manufacturer’s instructions. Pollen cDNA was primed with olig(dT) and cloned into the EcoRI/Xhol sites of the lambda HybriZAP vector (Stratagene, La Jolla, CA).

Root cDNA library was cloned into lambda gt10 (kind gift from M. Salanoubat). Both cDNA libraries were screened with a probe corresponding to exon 10 of the POK gene, with probes corresponding to exons 10 and 22 for the AtVps53 gene and with exons 11 and 15 for the AtVps54 gene.

Northern-Blot Analysis

Seed RNA was extracted following Downing (1992). RNA from other plant tissues was isolated as described in Procissi et al. (2003). Northern blots were performed on 20 µg of total RNA as described in Ausubel et al. (1990).

RT-PCR Experiments

cDNA synthesis was performed with Superscript RT (Life Technologies, Carlsbad, CA) from total RNA (3 µg) treated with DNase I, following the manufacturer’s protocol. cDNAs were amplified with 27 cycles of PCR. Primers used were: POK gene, POK-1 (5′-GGTTGAATGCGACATTC-3′) and POK-2 (5′-TCCACCAAGAAATCATCACAATAAAA-3′); P2 gene, P2-1 (5′-TCTAATGCGACATTC-3′) and P2-2 (5′-TCACAAAAAGA-AATCATCACAATACAA-3′); AtVps53 gene, 53 to 3 (5′-AAAACTCGAAATTGTTATCAATTGAC-3′) and 53 to 2 (5′-TCAAAGAATAATGCGACATTC-3′); and AtVps54 gene, 54 to 1 (5′-ATGGCTATCCCTATACATGA-3′) and 54 to 2 (5′-AAAAATTAGATGCGACATTC-3′).

5′ RACE Experiments

5′ RACE experiments were performed using a Life Technologies system on roots and flower bud RNA. Successively used primers were specific for exon 5 (5′-GCTTGAACATATAAGACAAAA-3′), the end of exon 4 (5′-CTAATGCCTAGACATCAATC-3′), and the beginning of exon 4 (5′-GTCACTTCTCTTTGATTTA-3′). RACE products were cloned into pGEM-T (Promega, Madison, WI).

GFP Constructs

The POK promoter region (2.2 kb) was amplified from Wassilewskia DNA using the primers 5′-TTTCTAGCGAGCGGCACTGAGCCTAGT-3′ and 5′-TCACCATGGTGCCCT-5′. The resulting fragment was cloned and sequenced into the pCR2.1-TOPO vector (Invitrogen, Carlsbad, CA). In parallel, the POK cDNA was amplified using the primers 5′-CTAAGTGGGGACTCTAAGCATC-3′ and 5′-GCTAATGCGACATTC-3′, allowing replacement of the stop codon by an Ncol restriction site at the 3′ end. The resulting PCR product was cloned and sequenced in the pCRBluntII-TOPO (Invitrogen). An Xbal/BglII promoter fragment and a BglII/Ncol cDNA fragment were isolated from the constructs described above and cloned together into the pTrc99A vector (Amann et al., 1988), where the promoter-cDNA junctions were verified. The resulting Xbal/Ncol fragment was cloned into pCambia1302 vector (CAMBIA GPO, Canberra, Australia) to produce the POK-GFP translational fusion.

Onion Epidermal Cell Bombardment

As described by Weigel and Glazebrook (2002), 7 µg of each construct were used to transform onion (Allium sativum) cells. Bombardments were performed with a PDS-1000/HE Biolistic Particle Delivery System (Bio-Rad Materials and Methods

Plant Material

The T-DNA transmission defect lines of Arabidopsis (L.) Heynh. ecotype Wassilewskia were isolated from the Versailles collection of T-DNA insertion mutants as described in Bonhomme et al. (1998b).

Pollen Tube Growth

In vitro growth of pollen tubes was tested as described in Procissi et al. (2001). In situ pollination and aniline blue staining were performed as described in Procissi et al. (2003).

GUS Assays

Histochemical GUS assays were performed as described in Procissi et al. (2003), except that K4Fe(CN)6 and K3Fe(CN)6 concentrations were increased to 2 mM for staining reproductive tissues.
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