

AtKP1, a kinesin-like protein, mainly localizes to mitochondria in *Arabidopsis thaliana*

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ABSTRACT

Kinesins and kinesin-like proteins (KLPs) constitute a large family of microtubule-based motors that play important roles in many fundamental cellular and developmental processes. To date, a number of kinesins or KLPs have been identified in plants including *Arabidopsis thaliana*. Here, a polyclonal antibody against AtKP1 (kinesin-like protein 1 in *A. thaliana*) was raised by injection the expressed AtKP1 specific C-terminal polypeptides in rabbits, and immunoblot analysis was conducted with the affinity-purified anti-AtKP1 antibody. The results indicated that this antibody recognized the AtKP1 fusion proteins expressed in *E. coli* and proteins of ~125 kDa in the soluble fractions of *Arabidopsis* extracts. The molecular weight was consistent with the calculated molecular weight based on deduced amino acids sequence of AtKP1. To acquire the subcellular localization of the protein, AtKP1 in *Arabidopsis* root cells was observed by indirect immunofluorescence microscopy. AtKP1 was localized to particle-like organelles in interphase or dividing cells, but not to mitotic microtubule arrays. Relatively more AtKP1 was found in isolated mitochondria fraction on immunoblot of the subcellular fractions. The AtKP1 protein could not be released following a 0.6 M KI washing, indicating that AtKP1 is tightly bind to mitochondria and might function associated with this kind of organelles.

Keywords: AtKP1, kinesin-like protein, protein expression, specific antibody preparation, localization, mitochondria.

INTRODUCTION

Kinesin and kinesin-like proteins (KLPs) constitute a superfamily of microtubule (MT) motor proteins that are found in all eukaryotic organisms. Members of the kinesin superfamily are known to play important roles in many fundamental cellular and developmental processes, such as intracellular transport of vesicles and organelles, spindle formation and elongation, chromosome segregation, MT dynamics, morphogenesis, and signal transduction [1-5]. All members of the kinesin superfamily have a highly con-

served catalytic region of about 350 amino acid residues, known as the motor domain. The motor domain in KLPs is located either in the N-terminus, C-terminus or in the middle of the protein. In addition to the motor domain, most KLPs have a stalk region and a highly variable tail, which is thought to interact with a specific cargo. Stalk and tail regions of different subfamilies of KLPs are not conserved [4].

In early 1990's, plant KLPs were firstly identified in pollen tubes of tobacco [6, 7]. To date, many KLP cDNA clones have been reported and characterized in several kinds of plants [4]. Systematic analysis of the sequences in *Arabidopsis* genome with the conserved motor domain of kinesins revealed the presence of 61 KLPs which are divided into three catalogues: N-terminal, C-terminal and internal motors according to the location of kinesin motor domain [8]. However, relatively little is known about their localization and functions [9]. *Kat* gene family (*katA*, *katB*, *katC*, *katD* and *katE*) was characterized in *Arabidopsis thaliana* [10-12]. *KatD* belongs to the C-terminal family but with a 240-amino acid stretch following the motor domain, and is a flower-specific motor protein [13]. AtKCBP (kinesin-like calmodulin-binding protein), a novel

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Abbreviations: FITC (fluorescein isothiocyanate); HEPES [(Na-N-(2-hydroxy-ethyl) piperazine-N'-2-ethanesulfonic acid)]; IPTG (isopropyl-1-thio- β -D-galactopyranoside); PIPES, (piperazine-N', N-bis 2-ethanesulfonic acid); PMSF (phenylmethylsulfonyl fluoride); TAME (p-tosyl-L-arginine-methyl-ester); TRITC (tetramethylrhodamine isothiocyanate).

KLP with a C-terminal motor domain, was isolated and characterized in *Arabidopsis* [14, 15]. Two kinesin-related proteins (AtPAKRP1 and AtPAKRP2) from *Arabidopsis* are phragmoplast-associated [16, 17]. In addition, another two *Arabidopsis* KLPs (AtMKRP1 and AtMKRP2) with mitochondrial targeting signals were found in mitochondria [18]. Recently, we have cloned a full-length cDNA (named as *AtKPI*, kinesin-like protein 1 in *A. thaliana*) (GenBank accession No. AF398149; AGI No. At3g44730), encoding a kinesin-like protein with high identity to KatD. Here we described the specific antibody raised in rabbit and the subcellular localization of AtKPI in *A. thaliana*.

MATERIALS AND METHODS

Materials

Seeds of *A. thaliana* (Columbia ecotype) were surface sterilized and germinated at 23°C on plates containing murashige and skoog (MS) media supplemental with 3% sucrose, 0.8% agar and 0.5 g/L [N-Morpholino]ethanesulfonic acid (Mes) (pH 5.7). After a further 7 d, seedlings were transplanted to soil and grown in a greenhouse at 23°C and 75% relative humidity.

Isolation of full-length *AtKPI* cDNA

Total RNA was extracted from *Arabidopsis* stems using TRIZOL reagent (Cat. #15596, Gibco, Grand Island, NY, USA) according to the manufacturer's description. First-strand cDNAs were synthesized with a kit from Promega (Madison, WI, USA), and then used as templates for PCR amplification with following primer pairs: forward primer Pkin1, 5'-ATC TTY GCA TAY GGA CAP AC-3', and reverse primer Pkin2, 5'-GTP TAI GGI ATG TCI TTI AGI TTY GAITG-3', which were designed based on the conserved amino acid sequence in the motor domain of kinesins as described [10].

The 3'RACE-PCR was carried out with the RNA PCR kit (AMV) ver. 3.0 (Cat. #DRR019, TaKaRa, Dalian, China). Briefly, the first round of PCR amplification was carried out only with the specific forward primer, 5'-GCT GGG TTC GTT TCT AAG GAA-3'. In the second round PCR, both the forward nested primer, 5'-GGC CAG ATC TGA TGA CC-3', and the adaptor primer were included in the mixture with the first round PCR products as templates. Finally, a 3,607 bp cDNA sequence was amplified by RT-PCR with the forward primer, 5'-CCC AAT TTA TTT CCG CGT-3' based on the genome sequence of *Arabidopsis* information from the GenBank, and the reverse primer, 5'-TGG GAC ACT CGT TCT TTA TGA-3' based on the sequence of the 3'-end RACE fragment. The full-length cDNA was cloned into pGEM T-easy vector and sequenced, which contains a stop codon (-72 bp site) upstream of candidate translational initiation codon, a poly A tail at the 3'-end, and an open-reading frame of 3,264 bp (denoted as *AtKPI*, *Arabidopsis* kinesin protein 1, accession number: AF398149; AGI number: At3g44730).

Expression vector construct

The coding region (3,264 bp) of AtKPI was PCR-amplified from the full-length *AtKPI* cDNA with following primer pairs: forward primer, 5'-GCG AAT TCT CTA GAA TGG ACC AAG GCG CGA TG-3' and reverse primer, 5'-GCG TCG ACT GGT ACC ATG AAC CTT GCA-3'. The PCR fragment was ligated into pGEM-T easy vector (Cat. #A1360, Promega, Madison, WI, USA) and se-

quence-confirmed. The plasmid was digested with *EcoR I/Sal I* and the recovered fragment was ligated into the *EcoR I/Sal I* predigested pET-30b(+) vector (Cat. # 69910-3, Novagen Inc., Madison, WI, USA), so as to a 6×His-tag coding sequence was fused to *AtKPI* at N-terminal to result a final construct of pET-AtKPI.

A 606 bp DNA fragment corresponding to nucleotides 2656 to 3261 of *AtKPI* full-length cDNA was PCR-amplified using the following primer pairs: forward primer, 5'-GCC ATA TGC TTC ACA CTG ATA ACT CTT CA-3'; reverse primer, 5'-GCG TCG ACT GGT ACC ATG AAC CTT GCA-3'. The PCR fragment was digested with *Nde I* and *Sal I* and ligated into the *Nde I-Sal I* predigested pET-30b(+) vector, so as to be in frame with the sequence encoding the histidine hexamer to form a final construct of pET-KA which will give a 28 kDa AtKPI-specific polypeptide as an antigen.

Expression and purification of recombinant proteins

The recombinant plasmids were transformed into *E. coli* BL21 (DE3) strain. The transformants were grown at 37°C to $A_{600} = 0.6$ OD and subsequently induced with 0.2 mM IPTG for 4 h at 25°C. The cells were harvested and sonicated on ice in buffer A (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM PMSF). The proteins from *E. coli* expressing AtKPI were suspended in 1×SDS sample buffer for SDS-PAGE and western blot analysis. The prokaryotic expressed AtKPI-specific polypeptide was affinity-purified with Ni-Chelating-Sepharose Fast Flow (Cat. #17-0575-01, Amersham Pharmacia, Uppsala, Sweden) as described by the manufacturers. All subsequent steps were performed at 4°C. After the bacteria cell extract was centrifuged at 12,000×g for 30 min, the resultant supernatant was applied to a self-packed 1ml Ni-resins pre-equilibrated with buffer A. After washing with 100 mM imidazole in buffer A, the His-tagged fusion protein was eluted with 300 mM imidazole in buffer A.

Preparation of anti-AtKPI polyclonal antibody

The purified His-tagged AtKPI-specific polypeptide was used as antigen to raise polyclonal antibodies in a female New Zealand white rabbit. Sera were passed through a DEAE-cellulose 52 column to obtain whole IgG. The antibodies specific to AtKPI were further affinity-purified by a blot purification method [19], and applied to immunoblotting or immunolocalization.

Protein extraction from plant tissues

Roots, stems, leaves, flowers, siliques (28-day-old) and young seedlings (7-day-old) of *Arabidopsis* were frozen and ground in liquid nitrogen. Proteins were extracted from tissues with a buffer containing 100 mM HEPES (pH 7.8), 5 mM EDTA, 5 mM EGTA, 10 mM Na_2VO_4 , 10 mM NaF, 50 mM glycerol-phosphate, 10 mM DTT, 5% glycerol, 1 mM PMSF, 10 mg/ml Leupeptin, 10 mg/ml Aprotinin, and 10 mg/ml Antipain on ice. The extract was then centrifuged at 20,000×g at 4°C for 40 min, and the supernatant were analyzed by SDS-PAGE and Western blot.

Subcellular fractionation

Organelles were isolated from *Arabidopsis* leaves by differential and sucrose density-gradient centrifugation as described by Laties [20] with some modification. All subsequent procedures were performed at 4°C. Leaves were homogenized with a tight-fitting homogenizer in ice-cold medium A consisting of 100 mM Tricine-KOH (pH 7.4), 300 mM sucrose, 10 mM KCl, 1 mM MgCl_2 , 1 mM potassium-EDTA, 0.1% BSA, 5 mM DTT, 1 mM PMSF, 10 mg/ml Leupeptin, 10

mg/ml Aprotinin, 10 mg/ml Antipain, 10 mg/ml pepstatin A, and 10 mg/ml TAME. The homogenate was filtered through four layers of cheesecloth and centrifuged at $2,600\times g$ for 15 min to pellet the cell debris and nuclei. The $2,600\times g$ -supernatant was spun for 15 min at $12,000\times g$ and the pellet was resuspended in medium B similar to medium A, but without DTT and at pH 7.0 instead of 7.4. About 2 ml of organelle suspension was layered on top of a sucrose gradient containing 6 ml of 0.6 M, 4 ml of 0.9 M, 8 ml of 1.2 M sucrose, 8 ml of 1.45 M and 8 ml of 1.8 M sucrose. The gradient was spun at $80,000\times g$ for 90 min. The fractions were collected and identified by measuring activities of marker enzymes, including cytochrome *c* oxidase for mitochondria, inosine 5'-diphosphatase (IDPase) for Golgi apparatus, catalase for peroxisomes, and chlorophyll for chloroplasts. Enzyme activities were determined by standard colorimetric methods [21]. Chlorophyll was determined using the protocol of Arnon [22]. The $12,000\times g$ supernatant was centrifuged for 40 min at $150,000\times g$ to isolate cytosol and microsomes according to the method of Khodjakov *et al* [23].

Extraction of peripheral proteins from mitochondria

Two of 300 μ l mitochondria fraction recovered from the sucrose density gradient were diluted 5-fold with medium B (mentioned as above) and spun at $12,000\times g$ for 15 min at 4°C to precipitate mitochondria. One of the mitochondria pellets was resuspended in 50 μ l 2 \times SDS sample buffer, the other was used for mitochondria peripheral protein isolation. For this, it was incubated for 30 min on ice with 30 μ l 0.6 M KI and centrifuged at $100,000\times g$ for 30 min at 4°C [24]. The KI-washed mitochondria supernatant was mixed with 20 μ l 5 \times SDS sample buffer and the KI-washed mitochondria pellet was resuspended in 50 μ l 2 \times SDS sample buffer. Equal volumes of mitochondria proteins, KI-washed supernatants, and KI-washed pellets were analyzed by immunoblotting.

Immunofluorescence localization

Immunofluorescence experiments were carried out according to Lee *et al* [17]. Root tips excised from 7-day-old seedlings were fixed in 4% paraformaldehyde plus 0.1% glutaraldehyde in PME (50 mM Pipes, 1 mM MgSO_4 , and 5 mM EGTA, pH 6.9) for 1 h, rinsed several times in PME and treated with 1% Cellulase "Onozuka" RS (R-10) (Yakult Honsha Co., Ltd., Tokyo) and 0.5% Pectolyase (Cat. # P5936, Sigma, St. Louis, MO, USA) in PME for 15 min. After rinsing, cells were released by squashing on slides coated with 1 mg/ml poly-L-lysine described previously by Palevitz [25]. After air-dried, the cells were treated with prechilled methanol for 6 min, followed by treatment with 0.5% Triton X-100 for 30 min and 1% BSA in PBS for another 30 min. The affinity-purified anti-AtKP1 antibody was applied at a 4-fold dilution in PBS (0.135 M NaCl, 2.7 mM KCl, 1.47 mM KH_2PO_4 , 8.1 mM Na_2HPO_4 , pH 7.3) in combination with a 400-fold dilution of anti- β -tubulin monoclonal antibody (Cat. #T5293, Sigma, St. Louis, MO) for 1 h at room temperature. Secondary antibodies were FITC-conjugated goat anti-rabbit IgG (Cat. #F0382, Sigma, St. Louis, MO) and TRITC-conjugated goat anti-mouse IgG (Cat. #115-025-003, Jackson ImmunoResearch Labs, West Grove, PA, USA), both diluted 100-fold. Images were acquired with a Confocal Laser Scanning Microscope (Bio-Rad, Hercules, CA).

SDS-PAGE and Western blot

Proteins were separated on SDS-PAGE according to Laemmli

[26], and then stained with Coomassie blue R_{250} or electroblotted onto nitrocellulose membrane (Hybond-c, Amersham) at 30V for 8 h followed by 50V for 40 min. After blocked in 5% nonfat milk in TBS (20 mM Tris-HCl, pH 7.5, 30 mM NaCl) supplemented with 0.05% Tween-20, the membranes were incubated with mouse anti-His monoclonal antibody (1:5000 dilution), or mouse anti-kinesin monoclonal antibody (Cat. #K1005, Sigma, 1:5000 dilution) or mouse anti- α -actin monoclonal antibody (Cat. #A5441, Sigma, 1:5000 dilution), or our purified rabbit anti-AtKP1 polyclonal antibody (1:40 dilution). Following three washes, the membranes were incubated with peroxidase-conjugated goat anti-mouse (or anti-rabbit) IgG (Cat. #115-035-003, #111-035-003, Jackson ImmunoResearch Labs, West Grove, PA; 1:5000 dilution). The membranes were visualized by using an enhanced Lumi-Light Western Blotting Substrate kit (Cat. #2015200, Roche, Indianapolis, IN, USA) and following the manufacturer's instruction.

RESULTS

Generation and specificity assay of a polyclonal antibody against AtKP1

Rabbit antiserum was raised using the prokaryotic ex-

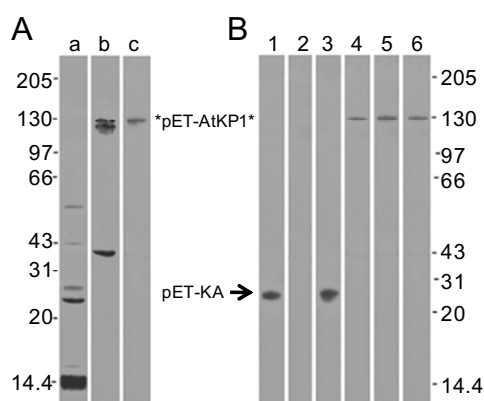


Fig. 1 Characterization of the anti-AtKP1 antibody by immunoblotting. **(A)** Proteins from Arabidopsis shoots were separated on a 7.5% SDS-PAGE gel (40 μ g protein per lane) and transferred onto nitrocellulose membrane. Total proteins on blots were probed with pre-serum (lane a), antiserum (lane b), and affinity-purified anti-AtKP1 antibody (lane c). **(B)** Western blot of the *E. coli* lysates containing either the His-tagged AtKP1-specific antigen or the entire AtKP1 polypeptides. Lanes 1 to 3 are the immunoblots of His-tagged AtKP1-specific polypeptide probed with monoclonal anti-6 \times histidine antibody (lane 1), monoclonal anti-kinesin antibody (lane 2), and affinity-purified anti-AtKP1 antibody (lane 3), respectively; Lanes 4 to 6 are the immunoblots of His-tagged AtKP1 probed with the antibodies including anti-6 \times histidine antibody (lane 4), kinesin antibody (lane 5), as well as our affinity-purified AtKP1 antibody (lane 6). Asterisks indicate the 125 kDa AtKP1 and the arrow indicates the 28 kDa AtKP1-specific polypeptide. The positions of molecular mass markers (in kilodaltons) are indicated on the left in **(A)** and on the right in **(B)**.

pressed His-tagged polypeptide (residues 886-1087, 28 kDa,) that is unique to AtKP1. The antibody was affinity-purified and its specificity was identified.

As shown in Fig. 1A, the antiserum reacts with several polypeptides in extract of whole *Arabidopsis* shoots (Fig. 1A, lane b), among which only the one with a molecular weight of 125 kDa, the size predicted from cDNA sequences of AtKP1, could be recognized by the affinity-purified anti-AtKP1 (Fig. 1A, lane c). To verify whether the 125 kDa polypeptide was AtKP1, we prepared the *E. coli* BL21 lysates containing the 28 kDa AtKP1-specific polypeptides encoded by pET-KA or His-tagged entire AtKP1 encoded by pET-AtKP1. When subjected to immunoblotting with monoclonal anti-6×histidine antibody (Fig. 1B, lanes 1 and 4), monoclonal anti-conventional kinesin antibody (Fig. 1B, lanes 2 and 5), and our affinity-purified anti-AtKP1 (Fig. 1B, lanes 3 and 6) respectively, the lysate containing the AtKP1-specific polypeptide produced a single immunoreactive band with a M_r of 28 kDa (Fig. 1B, lanes 1 and 3), corresponding to the specific polypeptides used as antigen that could not be recognized by the anti-conventional kinesin antibody (Fig. 1B, lane 2),

and the lysate containing the whole AtKP1 produced a 125 kDa bands (Fig. 1B, lanes 4-6). The results above indicate that our affinity-purified anti-AtKP1 antibody is specific for *Arabidopsis* AtKP1.

Detection of AtKP1 in different tissues

The expression of AtKP1 protein in *Arabidopsis* tissues was examined by immunoblot analysis with affinity-purified anti-AtKP1 (Fig. 2). A single band corresponding to the AtKP1 protein with a molecular size of 125 kDa was detected in total protein extract of seedlings, roots, stems, leaflets, flowers; but no such band was detected in siliques (Fig. 2B).

The relative amount of AtKP1 was quantitatively estimated for each organ. The samples were normalized, so that approximately equal amounts of total protein were loaded on a gel. When analyzed in immunoblotting, the blots were incubated with the antibodies against AtKP1-specific polypeptide and β -actin, so that the relative amount of AtKP1 from each organ could be determined using the relative amount of the β -actin as a reference point. The results show that AtKP1 was the highest enriched in flowers, and then in stems, leaves, roots in turn.

Immunofluorescence localization of AtKP1

Arabidopsis root tip is liable for immunofluorescent labeling, especially for the proteins in whole cells at different dividing phases. To get the distribution of AtKP1 in detail, single root tip cells at different phases of cell division were dually immunostained using affinity-purified AtKP1 antibody and anti- β -tubulin monoclonal antibody.

In interphase cells, AtKP1 was present throughout the cytoplasm (Fig. 3A-C). In preprophase cells, microtubules reorganized to form preprophase bands, but AtKP1 still localized to particle-like structures (Fig. 3D-F). In metaphase cells, no AtKP1 was detected on spindle microtubules (Fig. 3G-I). When microtubules concentrated to form phragmoplast, AtKP1 still keeps staying throughout the cytoplasm (Fig. 3J-L). The results demonstrate that AtKP1 does not correlate with mitotic microtubule organization as most other plant KLPs, but associates with some kinds of organelles.

Subcellular fractionation and identification

From the results of immunofluorescence microscopy, we know that AtKP1 is present on particle-like organelles in cytoplasm. In order to reveal the detail localization, we fractionated mitochondria, microsomes, chlorophyll, and cytosol from *Arabidopsis* leaves by sucrose density gradient ultracentrifugation and analyzed these subcellular fractions by Western blot with AtKP1 antibodies. As shown in Fig. 4A (lane 8), AtKP1 was only found in mitochondria

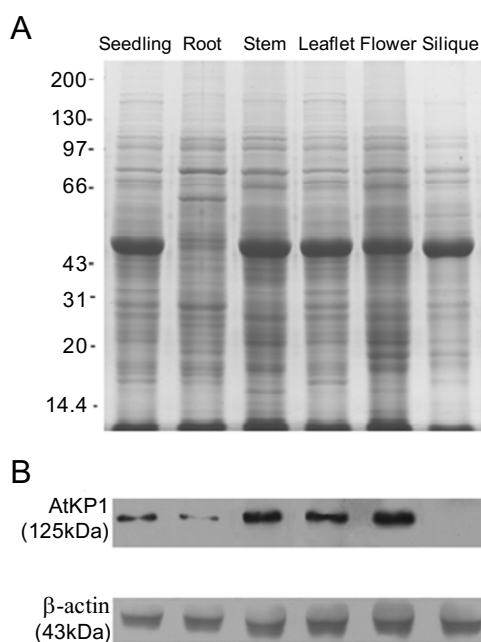


Fig. 2 The expression detection of AtKP1 in different tissues. **(A)** SDS-PAGE analysis of proteins from each organ of *Arabidopsis*. Each lane contains 40 μ g of total proteins extracted from seedlings, roots, stems, leaflets, flowers, and siliques. **(B)** Immunoblot analysis of AtKP1 with affinity-purified AtKP1 antibody to total proteins described as above. The lower panel shows anti- β -actin antibody labelling in the corresponding nitrocellulose membrane, confirming the presence of equal amounts of total proteins in each lane.

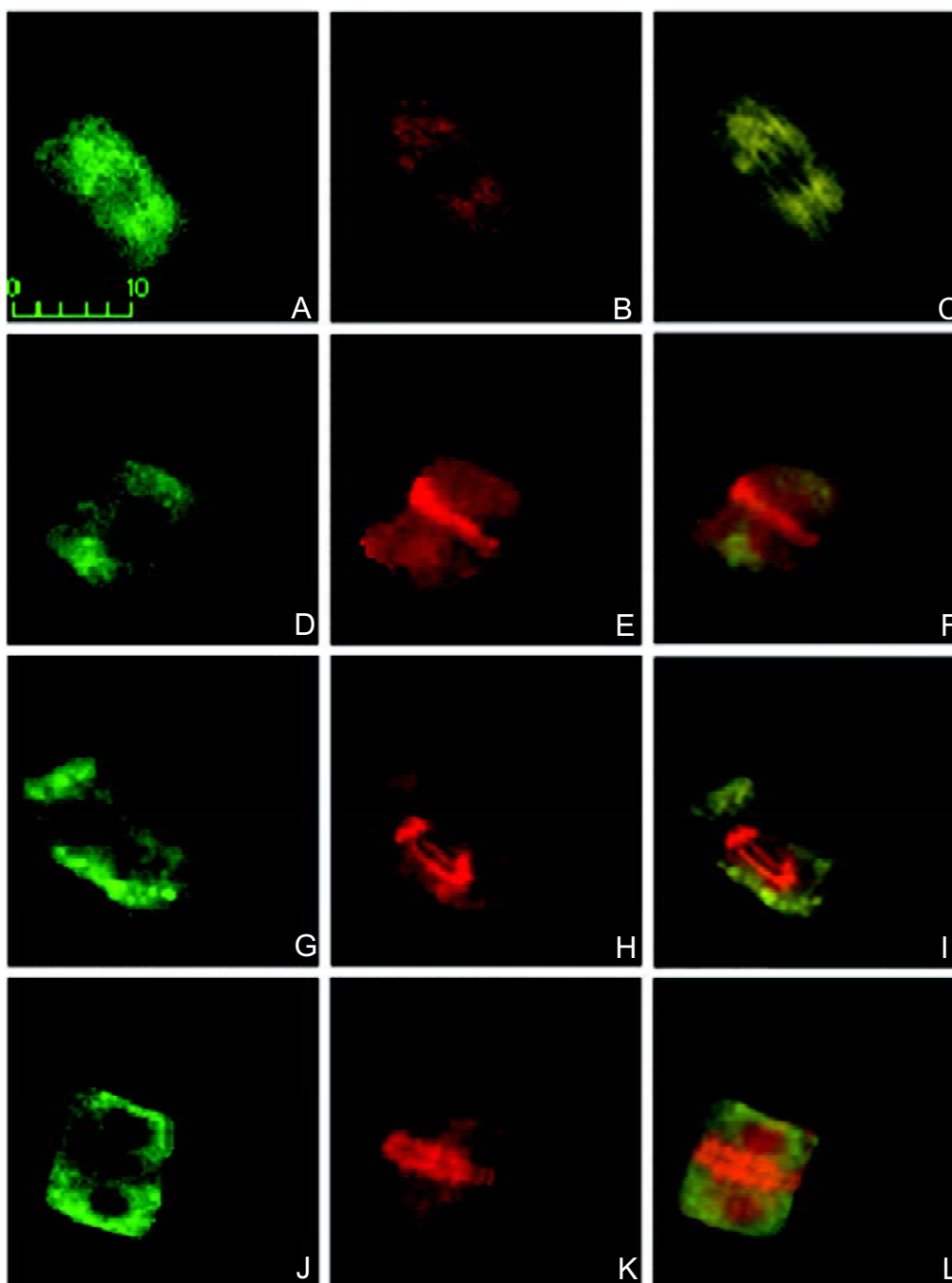


Fig. 3 Dual localization of AtKP1 and microtubules in root tip cells of *Arabidopsis*. Localization of AtKP1 stained with affinity-purified AtKP1 antibody (A, D, G, J), and microtubules stained with anti- β -tubulin antibody (B, E, H, K) are shown in root tip cells at interphase (A-C), preprophase (D-F), metaphase (G-I), and telophase (J-L). Composite images (C, F, I, L) are pseudocolored with AtKP1 shown in green and β -tubulin shown in red. Bars=10 μ m.

fraction determined by measuring the marker enzymes — cytochrom c oxidase for mitochondria, IDPase for Golgi apparatus, catalase for peroxisomes, and chlorophyll for chloroplast (Fig. 4B). When the isolated mitochondria were incubated in a 0.6 M KI solution, AtKP1 could not be re-

leased into the supernatant, indicating that AtKP1 is tightly bound to mitochondria (Fig. 4A, lane 12 and 13).

To know whether AtKP1 is also present in chloroplast, we specially isolated chloroplast from *Arabidopsis* leaves according to Ellis and Hartley [27]. The results from SDS-

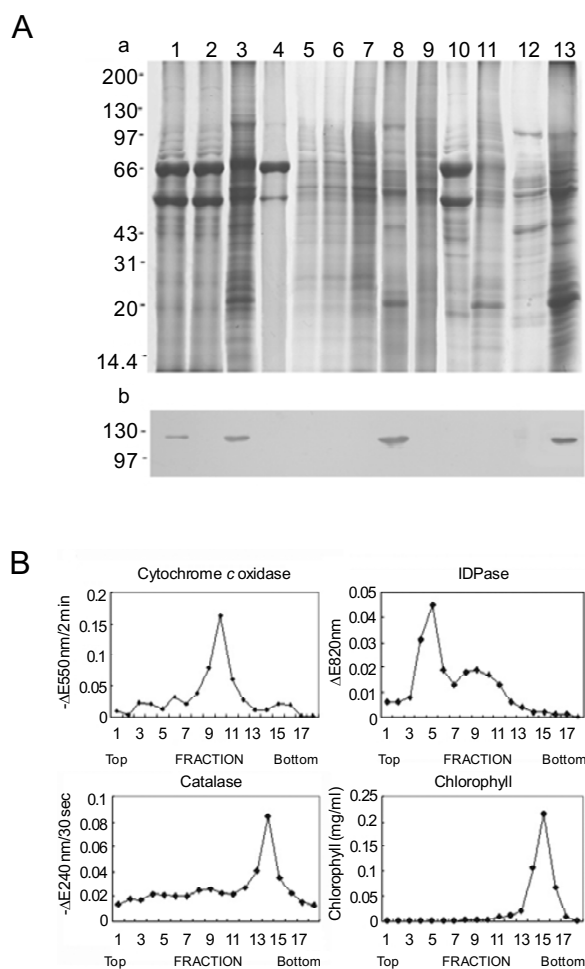


Fig. 4 Western blot analysis of AtKP1 in subcellular fractions isolated from *Arabidopsis* leaves. **(A)** Coomassie blue-stained gel (a) and western blots of subcellular fractions probed with the affinity-purified anti-AtKP1 antibody (only the region of the blot corresponding to the kinesin polypeptides is shown, b). The positions of the molecular mass markers (in kilodaltons) are indicated on the left. Lane 1, 2,600×g-supernatant (total homogenate); Lane 2, 12,000×g-supernatant; Lane 3, 12,000×g-pellet (crude mitochondria preparation); Lane 4, supernatant in sucrose density gradient; Lanes 5, 6, 7, 9, the subcellular fractions at the supernatant/0.6M, 0.6/0.9M, 0.9/1.2M, and 1.45/1.8M interzone in sucrose density gradient, respectively; Lane 8, mitochondria (the subcellular fraction at the 1.2/1.45M interzone in sucrose density gradient); Lane 10, cytosol; Lane 11, microsomes; Lane 12, KI-washed mitochondria supernatant; Lane 13, KI-washed mitochondria pellet. **(B)** Activity assay of cytochrome *c* oxidase, IDPase, catalase, and chlorophyll in subcellular fractions from *Arabidopsis* leaves. Fractions each in 2 ml were eluted from the sucrose density gradient and the activity of all marker enzymes was assayed. Fractions 5, 10, 14 shown in Fig. 4B represent subcellular fractions from the 0.6/0.9M, 1.2/1.45M and 1.45/1.8M interzones in sucrose density gradient, respectively.

PAGE and western blot indicated that no positive band corresponding AtKP1 was detected (data not shown).

DISCUSSION

We report here a specific localization of a kinesin-like protein AtKP1 to mitochondria of *Arabidopsis*. We took extensive precautions to ensure the specificity of our prepared antibody. First, the selection of AtKP1 specific region used as antigen was based on the Blast Similarity Searches at TAIR (<http://www.arabidopsis.org/blast>), and the antibody raised against the specific polypeptides was purified with affinity chromatograph method [19]. Second, we prepared the *E. coli* lysate containing AtKP1-specific polypeptide or entire AtKP1 to identify the specificity of the antibody. When subjected to immunoblotting with affinity-purified AtKP1 antibody, and another two kinds of commercial monoclonal antibodies including anti-6×histidine antibody and anti-kinesin antibody, both lysates produced only a single immunoreactive band with a molecular weight of 28 kDa or 125 kDa (Fig. 1B). No corresponding band was detected in lane 2 (Fig. 1B) for the reason that the commercial monoclonal anti-kinesin antibody could not recognize the unique polypeptides of AtKP1, demonstrating that our affinity-purified antibody is only specific to AtKP1. Immunoblots of *Arabidopsis* seedling extract showed that the antibody produced a single immunoreactive band (Fig. 1A, lane c), further indicating the high specificity of our affinity-purified AtKP1 antibody.

Arabidopsis root tip is liable for immunofluorescent labeling, because single whole cells in different mitotic phases are easily obtained. In isolated root tip cells, AtKP1 antibody stained organelle-like structures (Fig. 3) that were identified as mitochondria by subcellular fractionation and Western blot (Fig. 4). Mitochondria are cytoplasmic organelles of eukaryotic cells. Their morphology and dynamics are controlled genetically [28]. Several animal kinesin heavy chains or KLPs have been implicated in the movement of mitochondria, such as the N-terminal motors KIF1B and KIF5B in mouse cells [29, 30], and the KLP67A with N-terminal extension in early *Drosophila* embryos [31]. In plant cells, transport of mitochondria has been shown to rely on actin filaments [32, 33]. However, microtubules have also been shown to have a role in the positioning and/or tethering of mitochondria [33]. By using GFP (green fluorescence proteins) fusion and transient expression in tobacco leaf cells, Itoh *et al* identified two *Arabidopsis* proteins (MKRP1 and MKRP2, a new subclass of KLPs that contain an N-terminal mitochondrial targeting signal) within mitochondria where microtubules are absent, and suggested that the *Arabidopsis* MKRPs are involved in the segregation of mitochondrial nucleoids [18].

Many plant KLPs have been localized on preprophase band, mitosis spindle, or phragmoplast [10, 16, 34-37]. At least eight *Arabidopsis* KLPs are known to function in

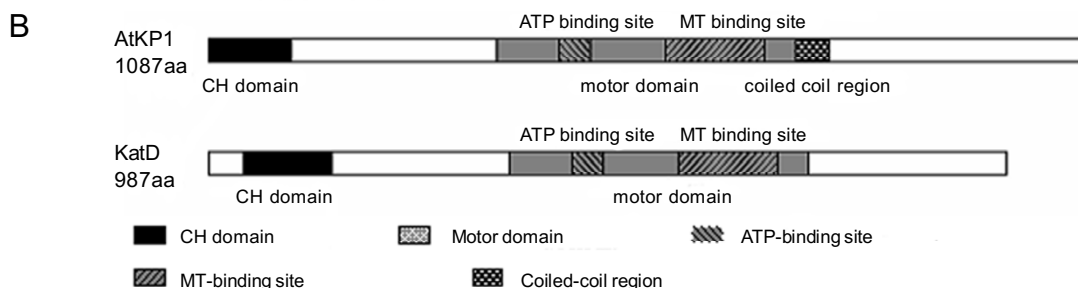
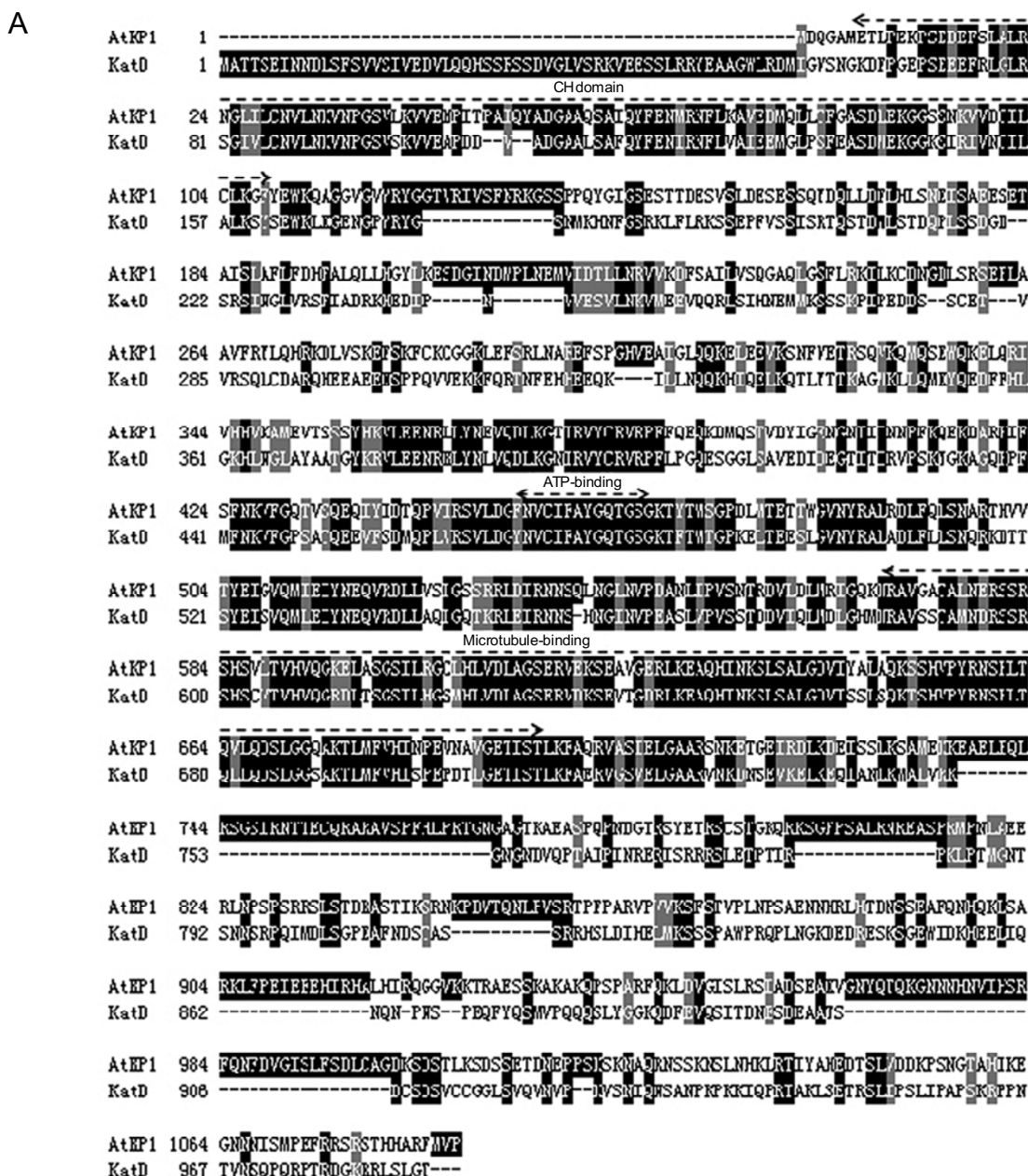


Fig. 5 Sequence and domain comparison of AtKP1 with KatD. **(A)** Sequence alignment of AtKP1 with KatD using BOXSHADE3.21 (http://www.ch.embnet.org/software/BOX_form.html). The identical amino acid residues for both sequences were highlighted with black background and the conserved amino acid residues were shaded. Gaps introduced into the sequences to optimize alignment are indicated by dashes. The CH-domain, ATP-binding consensus sequence and the putative microtubule (MT)-binding module are indicated by a dashed line above the sequences. **(B)** Schematic diagram for domain comparison between AtKP1 and KatD using SMART (<http://smart.embl-heidelberg.de/>), indicating that AtKP1 has a deduced coiled-coil region following its motor domain.

some aspects of cell division [4]. The results from our dual immunofluorescent labeling show that AtKP1 does not correlate with spindle function (Fig. 3). AtKP1 is widely expressed in seedlings, roots, stems, leaves and flowers, as ascertained with an antibody raised against specific polypeptides of AtKP1 (Fig. 2). Although AtKP1 and KatD [13] have similar structures: their motor domains share 70.9 % amino acid sequence identity, and their N-terminal regions have a similar single CH (calponin homology) domain which was originally found in actin-crossing link proteins [38]. (Fig. 5A), their expression patterns are very different. KatD is specifically expressed in flowers [13]. The detail domain comparison indicates that AtKP1 has a short coiled-coil region following its motor domain (Fig. 5B). The differences in expression patterns and domain structures suggest that AtKP1 and KatD might have different functions. AtKP1 was expressed in almost all Arabidopsis tissues, except in 28-day-old siliques (Fig. 2). One of the possible explanations is that AtKP1 might contribute mostly to some kind of tissues active in growth or development.

In summary, a new *Arabidopsis* KLP, AtKP1 with the highest similarity to KatD, is widely expressed in tissues, and tightly bind to mitochondria.

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