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TRH1 Encodes a Potassium Transporter Required for Tip Growth in Arabidopsis Root Hairs

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Root hair initiation involves the formation of a bulge at the basal end of the trichoblast by localized diffuse growth. Tip growth occurs subsequently at this initiation site and is accompanied by the establishment of a polarized cytoplasmic organization. Arabidopsis plants homozygous for a complete loss-of-function *tiny root hair 1* (*trh1*) mutation were generated by means of the T-DNA–tagging method. Trichoblasts of *trh1* plants form initiation sites but fail to undergo tip growth. A predicted primary structure of TRH1 indicates that it belongs to the AtKT/AtKUP/HAK K^+ transporter family. The proposed function of TRH1 as a K^+ transporter was confirmed in ^{86}Rb uptake experiments, which demonstrated that *trh1* plants are partially impaired in K^+ transport. In line with these results, TRH1 was able to complement the *trk1* potassium transporter mutant of Saccharomyces, which is defective in high-affinity K^+ uptake. Surprisingly, the *trh1* phenotype was not restored when mutant seedlings were grown at high external potassium concentrations. These data demonstrate that *TRH1* mediates K^+ transport in Arabidopsis roots and is responsible for specific K^+ translocation, which is essential for root hair elongation.

INTRODUCTION

Two fundamental parameters underlie development in higher plants: cell proliferation and differentiation. The coordinated action of these two parameters depends on the perception of and the response to an array of intracellular and extracellular developmental cues. To study cell expansion, we have chosen the epithelial layer of the Arabidopsis root. The outer cell layer of the seedling root, the epidermis, is composed of two distinct cell types that are arranged in files: hair-bearing and non-hair-bearing cells (Dolan et al., 1994; Scheres et al., 1994). Root hairs are tip-growing projections that emerge from specialized epidermal cells, the trichoblasts (Leavitt, 1904).

The development of root hairs in Arabidopsis can be divided into two phases: the early diffuse growth phase (initiation) and the later phase (tip growth); growth rates during these phases also differ (Dolan et al., 1994; Duckett et al., 1994). Tip growth is a form of polarized cell expansion found in fungi and in a number of cell types in plants (pollen tube and root hairs). This polarized growth of the root hair is due to the highly localized exocytosis of Golgi-derived vesicles and the deposition of cell membrane and wall material at a restricted area of the plasma membrane, the tip (Sievers and Schnepf, 1981). Genetic analysis of root hair growth has defined a number of genes involved in both the early and later phases of hair development. *RHD1* and *RHD6* are required for the earliest phases of root hair outgrowth (Schiefelbein and Somerville, 1990; Masucci and Schiefelbein, 1994), whereas *COW1*, *TIP1*, and *RHD3* are prerequisites for tip growth (Schiefelbein et al., 1993; Galway et al., 1997; Grierson et al., 1997; Ryan et al., 1997).

In Arabidopsis, initiation is sensitive to both Ca^{2+} and H^+ concentrations in the medium (Schiefelbein et al., 1992; Bibikova et al., 1998), suggesting a role for cytosol-free calcium concentration ($[Ca^{2+}]_i$) and pH in the transition to this developmental stage. Indeed, localized increases in $[Ca^{2+}]_i$ have been shown to precede the initiation of apical growth in some systems (Jaffe et al., 1974), although such increases in $[Ca^{2+}]_i$ have yet to be shown in root hairs (Wymer et al., 1997). Coordinated changes in cytosolic and apoplastic pH are clearly important for the initiation of root hair growth (Bibikova et al., 1998). Elongation is characterized by rapid tip growth ($\sim$2 μm/min), which becomes evident once the hair outgrowth is $\sim$20 μm in length. In this final stage, root hair growth is directed by a profound $[Ca^{2+}]_i$ gradient along the root hair axis (Wymer et al., 1997).

Root-hair growth assists the acquisition of mineral nutrients not only by increasing the surface of the root but also by exploring new undepleted soil layers. Among the mineral

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nutrients acquired by plants, potassium is the most abundant. Plant roots are able to accumulate K^+ to a level exceeding 100 mM from different types of soil. To adapt to the broad range of K^+ concentrations in soil, plants have evolved a "biphasic" mechanism of K^+ incorporation (Epstein et al., 1963). Low-affinity transport has been shown to provide a major transport pathway when the K^+ concentration in the soil is at the millimolar level, whereas high-affinity transport is vital for plants to sustain growth when external K^+ concentrations decrease to the micromolar range (Epstein et al., 1963; Epstein, 1966).

K^+ channel proteins have been demonstrated to be a molecular determinant of the low-affinity uptake system (Grabov and Böttger, 1994; Maathuis et al., 1997). In Arabidopsis, AKT1, a channel belonging to the Shaker family, is expressed predominantly in the root cell plasma membrane and has been shown to be responsible for K^+ uptake (Lagarde et al., 1996; Hirsch et al., 1998). Surprisingly, the AKT1 channel was also able to mediate potassium uptake from solutions that contained as little as 10 μM K^+ (Hirsch et al., 1998).

Electrophysiological studies indicate that the H^+-coupled high-affinity transport system operates in Arabidopsis roots (Maathuis and Sanders, 1994). However, no molecular details of K^+/H^+ transporters are available. Previous indications that the HKT1 transporter from wheat operates as a K^+/H^+ symporter (Schachtman and Schroeder, 1994) have not been confirmed in later experiments (Rubio et al., 1995). Alternatively, the sodium gradient across the plasma membrane was suggested to drive the high-affinity K^+ uptake system (Smith and Walker, 1989). It has been shown that free energy from the Na^+ gradient is used by the HKT1 transporter in wheat roots (Rubio et al., 1995). This mode of K^+ transport has not been confirmed, however, for AtHKT1, an HKT1 counterpart in Arabidopsis (Uozumi et al., 2000). Novel K^+ transporters belonging to the gene family described previously from *Schwanniomyces occidentalis* and *Escherichia coli* have been reported recently to mediate low-affinity, high-affinity, and dual-affinity K^+ transport (Quintero and Blatt, 1997; Fu and Luan, 1998; Kim et al., 1998; Rubio et al., 2000).

A key physiological role for K^+ lies in the osmotic balance of the plant cell, and K^+ transport across the vacuolar and plasma membranes contributes directly to turgor regulation. Because cell elongation is driven by turgor pressure, the operation of K^+ translocators is crucial for growth. This idea is strongly supported by recent studies of maize coleoptiles, in which auxin failed to stimulate cell elongation in the absence of K^+ in the medium or after the addition of K^+ channel blockers (Claussen et al., 1997). Moreover, a strong correlation was observed between K^+ channel expression and cell elongation after maize coleoptiles were stimulated by auxin (Philippar et al., 1999). The role of K^+ transport in root hair growth has yet to be analyzed.

To understand the molecular mechanism that determines root hair elongation, we have isolated a mutant impaired in root hair tip growth. This phenotype was designated *tiny root hair 1* (*trh1*). The mutant was generated by the T-DNA–tagging method, and the disrupted gene was demonstrated to encode a potassium transporter belonging to the AtKT/AtKUP/HAK family. In this study, we show that the TRH1 potassium uptake system is essential for root hair tip growth.

RESULTS

Mutant Phenotype of *trh1*

Root hairs grow in the differentiation zone of the root perpendicular to the root axis and can be characterized as polarized outgrowths from the basal end of trichoblasts (Figure 1). The *trh1* mutant was isolated from a population of 4000 mutagenized families containing T-DNA insertions (Feldmann, 1991). Consistent with the presence of a closely linked T-DNA insertion, each kanamycin-resistant plant segregated for the *trh1* mutation. A complete loss-of-function mutation in the *TRH1* gene results in the arrest of hair growth soon after initiation (Figure 1). The fact that TRH1 is required for the establishment of tip growth from the initiation site indicates that *TRH1* is a positive genetic regulator of tip growth. However, *trh1* trichoblasts occasionally develop multiple initiation sites (Figure 1), showing that the development of root hair growth from the basal end of the trichoblast may negatively regulate the initiation of other hairs in the same cell in a TRH1-dependent process. Segregation data from the F_2 progeny of a cross between homozygous mutants and the wild type indicate that *TRH1* represents a single Mendelian recessive allele (542 wild-type seedlings and 190 mutant seedlings; ratio 3:1, $\chi^2 = 0.36$, $P > 0.5$). Pollen tube growth is not defective in *trh1* plants, as might be expected, because this cell type also undergoes tip growth. Further detailed microscopic examination of *trh1* plants failed to identify any other cell growth defects, as were observed previously in *tip1* and *rhd3* mutants (Schiefelbein and Somerville, 1990; Schiefelbein et al., 1993; Galway et al., 1997; Grierson et al., 1997; Ryan et al., 1997).

Isolation of the *TRH1* Gene

To determine the copy number of T-DNA inserts, we hybridized T-DNA sequences to genomic DNA from the Arabidopsis mutant. The DNA gel blot showed two hybridizing bands, indicating that two T-DNAs were present (Figure 2). By plasmid rescue, it was possible to isolate two plant DNA fragments flanking the T-DNA insertions: one 200 bp in length and the other 1.3 kb in length. Both fragments hybridized to a single 1.5-kb EcoRI genomic fragment of wild-type plants, indicating that both T-DNA copies were introduced into the same locus. The 1.3-kb fragment isolated from plasmid res-

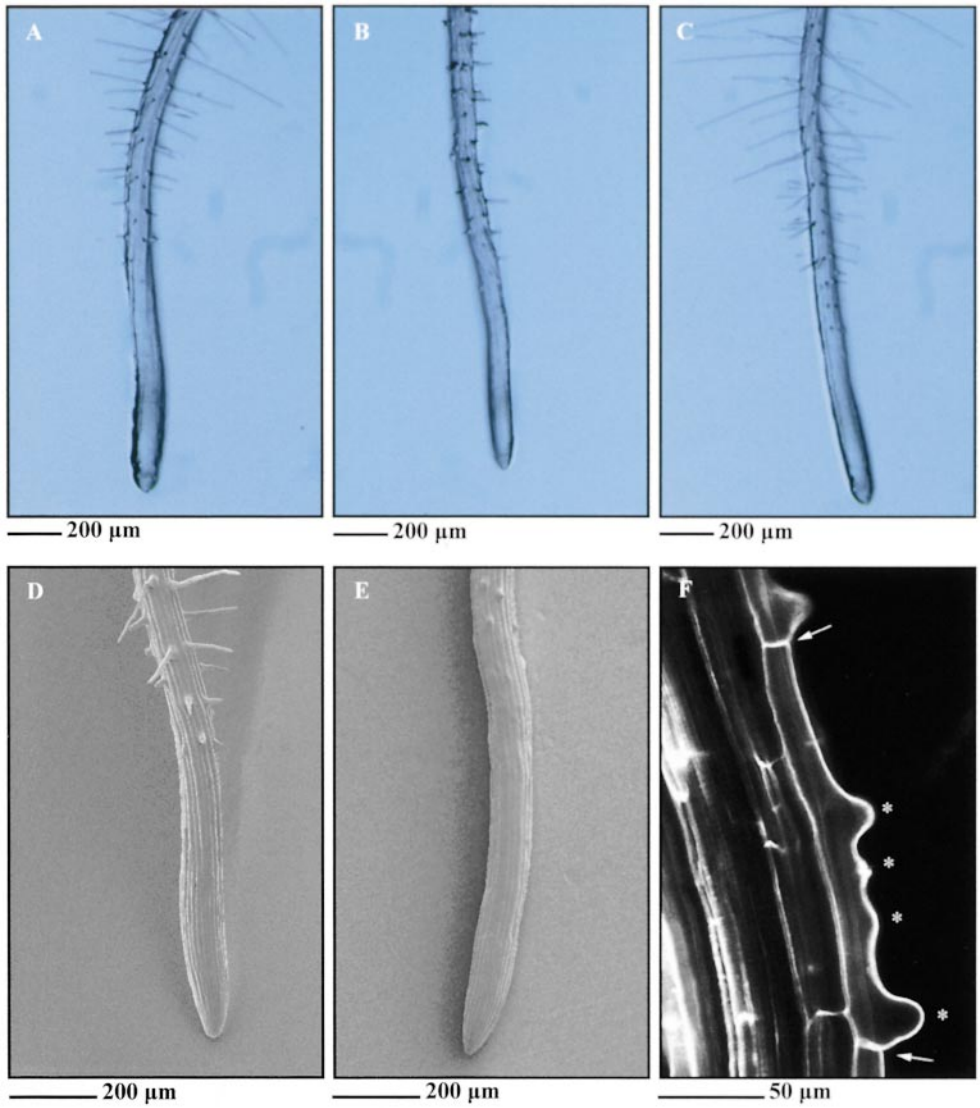

Figure 1. Phenotypes of Wassilewskija Wild Type, *trh1* Mutant, and *trh1* Transformed with the *TRH1* Gene.

(A) and **(B)** Wild-type **(A)** and *trh1-1* **(B)** primary roots from 1-week-old seedlings.
(C) *trh1* mutant transformed with the 8.5-kb BamHI-SalI genomic fragment containing the *TRH1* gene. The same phenotype was observed when *trh1* mutant plants were transformed with the 7.1- and 6.6-kb fragments.
(D) and **(E)** Scanning electron micrographs of wild-type **(D)** and *trh1* **(E)** primary root hair cells.
(F) Confocal micrograph of *trh1* primary root hair cells. Asterisks show multiple sites of root hair initiation in a single root hair cell that is indicated by the arrows.

cue was used to screen an Arabidopsis genomic library. Eight overlapping genomic clones were identified that spanned the 35-kb region shown in Figure 3. All clones were also positive when counterscreened with the 200-bp rescued fragment. Four identical cDNA clones were isolated from the cDNA library when screened with the 1.5-kb EcoRI fragment, indicating that it contained transcribed sequences.

DNA gel blot analysis of the isolated genomic clones showed that the isolated cDNA hybridized to an 8.5-kb BamHI-SalI fragment from the positive clone λ-72 (Figure 3). The cDNA and ~5 kb from the genomic clone were sequenced and shown to represent the same sequences. The cDNA was truncated in the 5′ coding region because no initiation codon was present. An expressed sequence tag

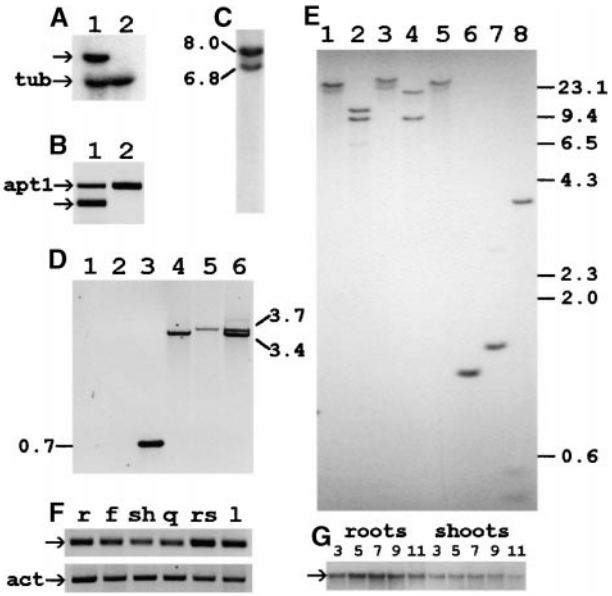

Figure 2. Expression and Copy Number of the *TRH1* Gene.

(A) RNA gel blot analysis. Approximately 30 μg of total RNA was loaded from wild-type (lane 1) and *trh1* (lane 2) 1-week-old roots. The *TRH1* (arrow) and tubulin (tub) gene transcripts detected are shown.

(B) Reverse transcription–polymerase chain reaction (RT-PCR) analysis of RNA isolated from wild-type (lane 1) and mutant (lane 2) plants. The specific primers used for the *TRH1* gene (exon2 and st2rev; arrow) generated a fragment of 250 bp. Primers specific to the *APT1* gene generated a fragment of 478 bp (apt1) and were used as a control.

(C) T-DNA copy number. Genomic DNA from *trh1* mutant plants was digested with EcoRI, and the resulting DNA gel blot was hybridized to T-DNA. The sizes of the two hybridizing bands are in kilobases.

(D) Two T-DNA copies were inserted into the first intron of the *TRH1* gene as an inverted dimer. PCR analysis of the genomic DNA isolated from wild-type (lanes 1 to 3) or *trh1* mutant (lanes 4 to 6) plants was performed. The combinations of primers used are as follows: lanes 1 and 4, st2rev/#1242; lanes 2 and 5, exon2/#1242; lanes 3 and 6, exon2/#1242/st2rev. Numbers denote the lengths of the PCR products in kilobases.

(E) Arabidopsis genomic DNA was isolated from ecotype Columbia and digested with XhoI, BamHI, SalI, EcoRI, HindIII, and PstI (lanes 3 to 8, respectively) and XhoI-SalI (lane 1) or BamHI-SalI (lane 2). The numbers at right are in kilobases.

(F) Quantitative RT-PCR analysis of the *TRH1* gene performed with RNA isolated from roots (r), flowers (f), shoots (sh), siliques (q), rosette leaves (rs), or leaves (l). The RT-PCR products for *TRH1* (arrow) and actin (act) are shown. Actin was used as an internal control.

(G) RNA gel blot analysis of the *TRH1* gene transcripts during seedling development. Wild-type Arabidopsis seedlings at 3, 5, 7, 9, and 11 days after germination were dissected, and RNA was isolated from roots and shoots. Each lane contains 20 μg of total RNA. The arrow denotes the *TRH1* transcript.

(EST) entry in the Arabidopsis database (H10G7) was completely identical at the 3′ end to the isolated cDNA. This EST is a full-length cDNA that contained the initiation codon and the 5′ untranslated region. Alignment of the sequences from the EST, the isolated cDNA, and the genomic region showed that the *TRH1* gene consists of nine exons separated by eight introns ranging from 71 to 430 bp in length. The longer exon is located at the 3′ end of the gene. The first exon consists of only the initiation codon.

Sequence and polymerase chain reaction (PCR) analysis of the genomic DNA from the *trh1* plant showed that both T-DNAs had been inserted in the same position within the first intron of the gene in a tail-to-tail orientation. This result indicates that only the isolated gene corresponds to the *trh1* phenotype. One of the T-DNAs is complete and the other is truncated (Figures 2 and 3). The gene is localized on chromosome IV (*Arabidopsis thaliana* ESSAII Sequencing Project, bacterial artificial chromosome clone F9D16; GenBank accession number AL035394). The *TRH1* cDNA consists of a single 2325-nucleotide open reading frame encoding a predicted protein of 775 amino acid residues with a molecular mass of 86,842 D (Figure 4). Sequence analysis identified a region similar to a putative cAMP-dependent protein kinase phosphorylation site at amino acid 730 and some other phosphorylation sites.

The *TRH1* Gene Complements the *trh1* Mutant Phenotype

To establish whether the disrupted gene is responsible for the *trh1* mutant phenotype, we hybridized the *TRH1* gene to total RNA isolated from mutant and wild-type plants. RNA gel blot analysis showed that the mRNA transcribed from the *TRH1* gene was not detected in the *trh1* plants (Figure 2). A more detailed analysis with reverse transcription (RT)–PCR verified this result (Figure 2). Furthermore, we transformed mutant plants with genomic DNAs spanning the *TRH1* gene. Five different wild-type genomic fragments were used: the 8.5-kb BamHI-SalI fragment and the 7.1-, 6.6-, and 5.7-kb fragments containing ~2.7-kb, 1800-bp, 1300-bp, and 300-bp upstream sequences, respectively (Figure 3). The upstream fragment, from −3.1 kb to +310 bp, was also used for complementation analysis (Figure 3). The 8.5-kb BamHI-SalI fragment and the 7.1- and 6.6-kb fragments (Figure 3) complemented the *trh1* phenotype, producing plants with wild-type root hairs (Figure 1). These results clearly demonstrate that the *TRH1* gene is required for the establishment of tip growth from the initiation site of the root hair cell. The 5.7-kb fragment (Figure 3) containing ~300-bp upstream sequences failed to complement the mutant phenotype. The 300 nucleotides of the promoter region therefore were insufficient for proper expression of the *TRH1* gene. The fragment containing exclusively upstream sequences was also unable to complement the mutant plant (Figure 3).

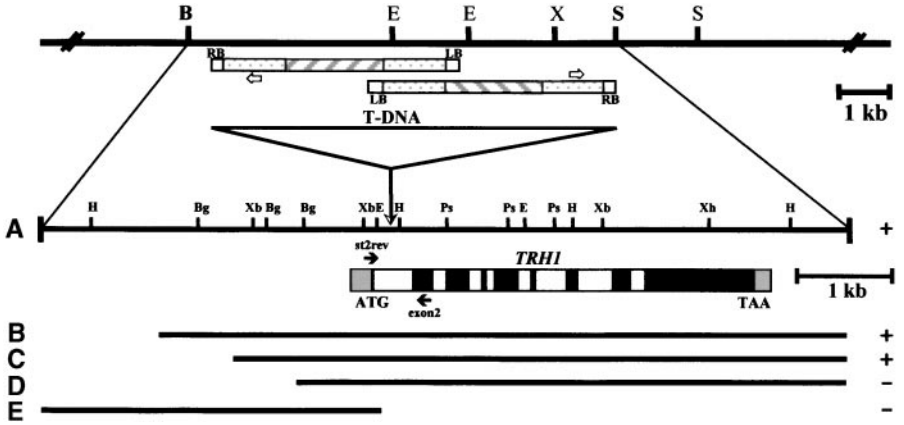

Figure 3. The *TRH1* Locus.

The genomic region of ~35 kb of contiguous DNA was isolated from a Columbia genomic library using as a probe the genomic fragment (1.3 kb) that was identified by plasmid rescue of the *trh1* mutant plants. Five fragments were used for transformation of mutant plants. The 8.5-kb **(A)**, 7.1-kb **(B)**, and 6.6-kb **(C)** fragments converted the mutant phenotype to wild type (+). The 5.7-kb **(D)** and the upstream 3.5-kb **(E)** fragments did not restore the phenotype (−). The T-DNA insertion site is depicted by the triangle within the first intron. Two T-DNA copies were inserted in the 1.5-kb EcoRI fragment in an inverted orientation. Black boxes represent exons, white boxes represent introns, and gray boxes represent 5′ or 3′ untranslated cDNA sequences. First and last codons are shown. Solid arrows st2rev and exon2 denote the specific primers for the TRH1 gene in the first and second exons, respectively. The open arrows denote the specific primer #1242 from pBR322. RB and LB, left and right border, respectively. The restriction map of the region *TRH1* is also shown. B, BamHI; Bg, BglII; E, EcoRI; H, HindIII; Ps, PstI; S, SalI; X or Xh, XhoI; Xb, XbaI. Hatched boxes represent T-DNA structure. Stippled boxes represent pBR322 sequences.

TRH1 Gene Expression

Based on high-stringency DNA gel blot analysis, we determined that only one copy of the *TRH1* gene is present in the Arabidopsis genome, because one major hybridizing band was generated by the restriction enzymes EcoRI, HindIII, SalI, and PstI (Figure 2). Identical hybridization patterns of genomic DNA isolated from Columbia or Wassilewskija ecotypes were observed. To determine the expression pattern of the *TRH1* gene, we isolated RNA from the aerial parts (leaves, stems, flowers, rosette leaves) and from the root of the plant. Quantitative RT-PCR analysis showed that the *TRH1* gene is expressed almost equally in all parts of the plant (Figure 2). To investigate the temporal expression and developmental accumulation of the *TRH1* transcripts during the early stages of plant development, we isolated RNA from roots and shoots of developing seedlings. RNA gel blot analysis showed that during seedling development, the levels of *TRH1* gene transcripts were higher at the early stages of seedling growth and that root tissues accumulated higher levels of these transcripts compared with the aerial parts (Figure 2).

TRH1 Is Similar to a Family of Potassium Transporters

Alignment of TRH1 showed that the predicted polypeptide shares significant homology with the bacterial KUP1 (Schleyer and Bakker, 1993) and yeast HAK1 (Banuelos et al., 1995) potassium transporters. This alignment yielded 30 and 26% identity overall for KUP1 and HAK1, respectively. The alignment of the predicted amino acids of TRH1 yielded an average 46% identity with the two other isolated members of the potassium transporter gene family from Arabidopsis, AtKT1 and AtKT2 (Quintero and Blatt, 1997; Fu and Luan, 1998; Kim et al., 1998) and 33% identity with HvHAK1 from barley (Santa-Maria et al., 1997). However, the similarity of TRH1 to the other two members of the gene family AtKT1 and AtKT2 is ~66% (Figure 4).

The polypeptide deduced from the *TRH1* gene can be broadly categorized into three distinct domains (Figure 5). The N- and C-terminal domains, consisting of 7 and 204 amino acids, respectively, are hydrophilic, and the middle domain, consisting of 564 amino acids, is mostly hydrophobic. By computer analysis of the deduced polypeptide, TRH1 was predicted to contain 14 transmembrane domains. The number of hydrophilic amino acids separating the transmembrane spans ranges from 3 to 73, with an average length of ~20 amino acids. The largest hydrophilic loop is located between the second and third transmembrane spans (Figure 5). This position is consistent with those predicted for KUP1 from bacteria, HAK1 from yeast, and AtKT1 and AtKT2 from Arabidopsis (Schleyer and Bakker, 1993; Banuelos et al., 1995; Quintero and Blatt, 1997; Kim et al., 1998).

To ascertain the effect of external potassium concentrations on the growth of root hairs, we grew Arabidopsis seedlings

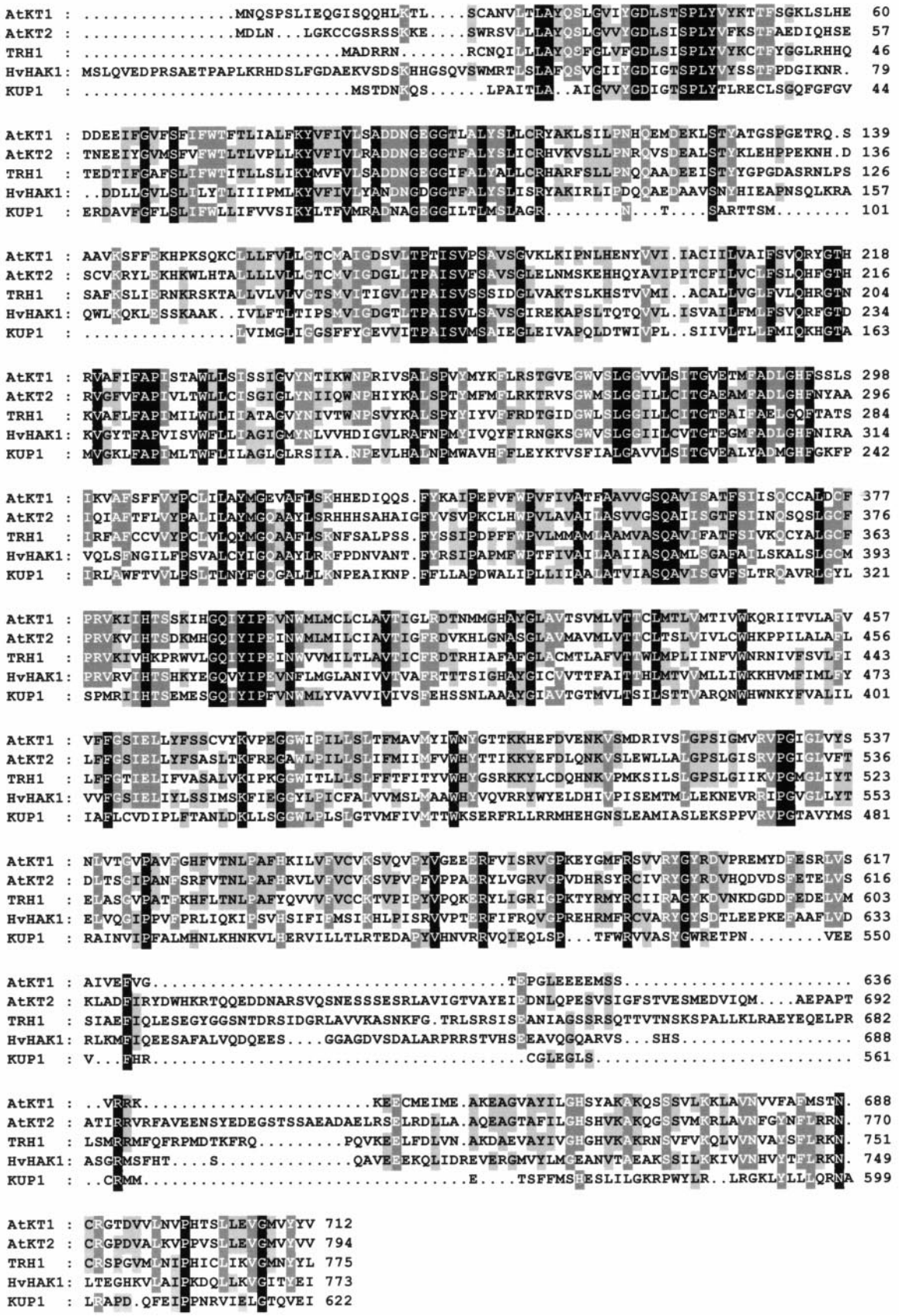

Figure 4. Alignment of K^+ Transporters.

on medium containing various concentrations of K^+. The *trh1* phenotype was prominent from low (0.1 mM) to high (50 mM) external potassium concentrations. Similarly, there was very little effect on root hair growth in wild-type plants grown at various concentrations of external potassium. To determine whether TRH1 functions in potassium uptake, we performed K^+ uptake analysis using ^{86}Rb as a radioactive tracer. Wild-type plants accumulated ^{86}Rb at a rate of 3.7 nmol/hr (calculated from data shown in Figure 6 and normalized per 1 g of fresh weight and 1 μmol of Rb in the incubation medium). Similar results (4.5 nmol/hr) were obtained by Hirsch et al. (1998) under similar experimental conditions (10 μmol of alkali cations in the medium). A significantly lower rate of ^{86}Rb accumulation was detectable in the *trh1* mutant after only 10 min of incubation. After 2 hr of incubation, the normalized rate of ^{86}Rb uptake by *trh1* was 2.2 nmol/hr, which was 40% less than the uptake rate in wild-type plants.

TRH1 Complements Yeast Mutants Defective in High-Affinity K^+ Transport

To analyze further TRH1 function as a potassium transporter, we assessed its capacity to rescue yeast mutants defective in K^+ transport. Two yeast strains were used: the single mutant strain M398 with the high-affinity potassium transporter *TRK1* gene deleted and the double mutant strain CY162 lacking both potassium transporter *TRK1* and *TRK2* genes (Gaber et al., 1988; Ko et al., 1990; Ko and Gaber, 1991). Both mutant strains carrying the potassium transporter deletions are lethal when grown in medium with low levels of potassium (Anderson et al., 1992). We transformed both strains with the expression vector and the expression vector containing the *TRH1* cDNA. The *TRH1* cDNA failed to complement the CY162 yeast strain carrying *trk1*Δ*trk2*Δ deletions because the transformants were unable to grow in medium containing low potassium concentrations (2 or 0.2 mM). However, the yeast strain M398 transformed with the *TRH1* cDNA grew like the wild-type yeast strain in medium containing potassium concentrations as low as 0.2 mM (Figure 7). This finding suggests that the *TRH1* gene product possesses high-affinity potassium transporter activity in yeast cells.

To determine the functional domains of the TRH1 polypeptide, we deleted the hydrophilic C-terminal end; the remaining truncated *TRH1* gene was used for complementation experiments of the M398 yeast strain. This truncated construct, which contained all transmembrane domains but lacked the putative cAMP-dependent kinase phosphorylation site, was unable to complement *trk1*Δ deletion of the M398 yeast strain (data not shown). This result indicates that the hydrophilic C-terminal end is a key feature of high-affinity potassium transporter activity. Alternatively, this hydrophilic end may be important in the correct deposition and localization of the protein.

DISCUSSION

TRH1 Gene Disruption Arrests Root Hair Tip Growth

Several genes that disrupt the initiation of root hair development, tip growth, hair morphology, and the spatial arrangement of root hairs have been identified and isolated from Arabidopsis. The *TRH1* gene is required for the establishment of tip growth from the initiation site, suggesting that it acts as a positive genetic regulator of tip growth. Trichoblasts of *trh1* plants, however, occasionally develop multiple initiation sites, indicating that either the TRH1 gene product itself or the outgrowth from the basal end of the trichoblast may inhibit any initiation of additional hairs from the same cell. Mutants impaired in patterning and cellular differentiation, such as *ttg* and *gl2*, cause root hairs to form on nearly every root epidermal cell. This implies that *TTG* (a MYC transcription factor) and *GL2* (a homeobox gene) either promote nonhair differentiation or repress root hair differentiation (Galway et al., 1994; Masucci et al., 1996). A novel gene (*WER*), which encodes a MYB-type protein, is also required for nonhair fate (Lee and Schiefelbein, 1999). The *CPC* gene, which encodes a protein with a MYB-like DNA binding domain but no typical transcriptional activation domain, is required for hair/cell differentiation (Wada et al., 1997). Other genes that affect tip growth, such as *RHD1* and *RHD6*, are required for the early stages of root hair diffuse growth and also affect pollen tube formation (Schiefelbein and Somerville, 1990; Masucci and Schiefelbein, 1994; van Den Berg et al., 1998). The *COW1*, *TIP1*, and *RHD3* genes are important for tip growth but also affect the morphology of root hair outgrowth (Galway et al., 1997; Grierson et al., 1997; Ryan et al., 1997). Plants homozygous for point mutations in the *RHD2* gene initiate root hairs, which cease growing almost immediately (Schiefelbein and Somerville, 1990). This phenotype is similar to that of the *trh1* mutant. Both

Figure 4. (continued).

Alignment of the deduced amino acid sequences of TRH1 with bacterial KUP1 and plant HvHAK1 from barley (Schleyer and Bakker, 1993; Santa-Maria et al., 1997) and AtKT1 and AtKT2 from Arabidopsis (Quintero and Blatt, 1997; Kim et al., 1998). One-letter amino acid code is used. Dots have been introduced to optimize sequence alignment. Identical or similar amino acids are shaded black or gray, respectively. The GenBank accession numbers of the complete *TRH1* cDNA and genomic sequences are AJ296155 and AJ296156, respectively.

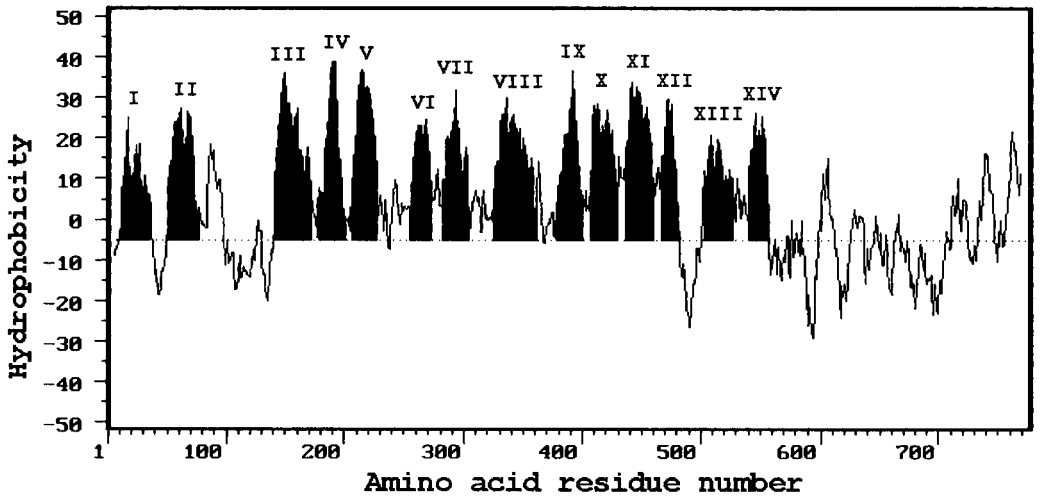

Figure 5. Hydropathy Profile of Amino Acid Sequences Deduced from the *TRH1* Gene.

Hydropathy indices were calculated using an interval of 13 amino acids. Roman numerals denote the number of transmembrane (shaded) spans.

trh1 and *rhd2* homozygous plants have normal appearance and exhibit no other morphological abnormalities. However, they are not alleles, because *rhd2* mapped on chromosome V (Aeschbacher et al., 1994) and *trh1* mapped on chromosome IV. The cessation of growth in *rhd2* plants may be in part the result of defective Ca^{2+} gradient formation in the root hairs, because RHD2 is required for calcium gradient formation in the tip of the growing hair. It is possible that RHD2 may be required for the activity of TRH1. Determining the activity of TRH1 in plants homozygous for *rhd2* will be instructive.

TRH1 Is a Potassium Transporter

TRH1 shares considerable amino acid sequence homology with the *KUP1* and *HAK1* genes, which are potassium transporters from Escherichia and Schwanniomyces, respectively (Schleyer and Bakker, 1993; Banuelos et al., 1995). This fact suggests that TRH1 can function as a K^+ transporter in Arabidopsis. This hypothesis has been confirmed in uptake experiments in which ^{86}Rb was used as a radioactive tracer. The rate of K^+ transport was reduced significantly (40%) in *trh1* plants (Figure 6). We cannot exclude, however, that this reduction was partially due to smaller surface area in *trh1* roots. The mutant plants did not develop symptoms of K^+ starvation. Thus, we conclude that the rate of K^+ transport was not a limiting factor for whole-plant growth in our experiments, probably because the total K^+ concentration in Murashige and Skoog (1962) medium is as high as 20 mM.

In accordance with the proposed K^+ transport function, the *TRH1* gene complemented the M398 yeast strain deficient for the high-affinity K^+ uptake system (Gaber et al., 1988). These results indicate that the *TRH1* gene encodes a high-affinity potassium transporter, at least when expressed in yeast. The *TRH1* gene is a member of the *AtKT/AtKUP/HAK* gene family (Quintero and Blatt, 1997; Fu and Luan, 1998; Kim et al., 1998). Another protein from the same family, AtKT1 (AtKUP1), has been reported to exhibit high-affinity (Kim et al., 1998) or dual-affinity (Fu and Luan, 1998) potassium transport activity. The *AtKT2* (*AtKUP2*) gene was suggested to mediate low-affinity K^+ transport, with K_m = 40 ± 10 mM (Quintero and Blatt, 1997).

Although *TRH1* is a member of the *AtKUP* gene family, DNA gel blot analysis revealed no cross-hybridization with any genomic fragments of the other *AtKUP* genes, indicating a high degree of divergence within this gene family. *TRH1* is constitutively expressed in most organs of Arabidopsis. The developmental pattern of mRNA accumulation during the early stages of postembryonic root growth showed almost constant levels. However, plants homozygous for the loss of function of this gene showed no other phenotype except defective root hair growth. The *AtKUP* gene family exhibits similar patterns of gene expression (Kim et al., 1998). This result also suggests that the function of each product of the *AtKUP* gene family may depend on additional factors (i.e., β subunits) or on post-translational modification (i.e., phosphorylation).

The AtKUP1 transporter from Arabidopsis was not expressed in roots (Kim et al., 1998) but it was detected in shoots, implying that the function of TRH1 may be redundant in other plant tissues or organs. We also cannot exclude the possibility that other potassium transporters from

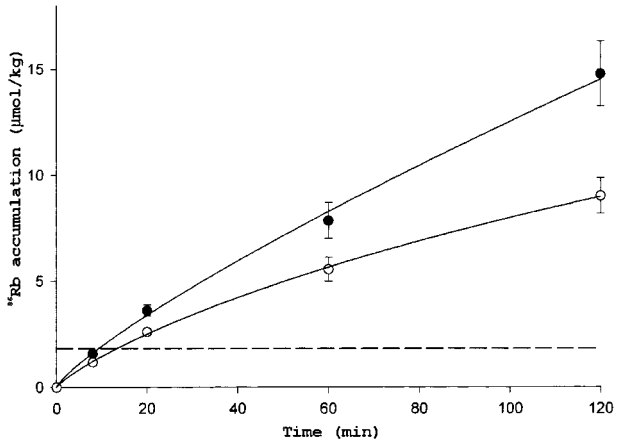

Figure 6. Time Dependence of ^{86}Rb Uptake by Wild-Type and *trh1* Plants.

Each point represents the mean ±SE for nine seedlings. The dashed line indicates a concentration of ^{86}Rb in the incubation medium. Solid circles, wild type; open circles, *trh1*.

the same family are active in roots. This complexity in affinity and redundancy may reflect plant plasticity in adaptation to soil environments with different levels of K^+.

Other potassium translocators preferentially present in root tissues are the inward- and outward-rectifying K^+ channels AKT1 and SKOR, respectively (Lagarde et al., 1996; Gaymard et al., 1998). AKT1 is localized in the plasma membrane of root cells, and the *SKOR* gene is expressed in the root pericycle and in stelar parenchyma cells. SKOR is involved in K^+ release into the xylem sap, and its disruption results in decreased K^+ translocation toward the shoots (Gaymard et al., 1998). Therefore, it is reasonable to believe that potassium is transported from the rhizosphere by the AKT1 localized in the plasma membrane of the root cells and secreted to the xylem sap by the SKOR for transport to other parts of the plant. A place for TRH1 in the K^+ transport pathway has yet to be identified, but its significance for root hair development suggests that this transporter may be important for specific localized K^+ delivery.

Predicted Protein Structure

Computer analysis showed that the *TRH1* gene product is composed of a hydrophobic part consisting of 14 transmembrane domains, in contrast to other members of the AtKT/AtKUP/HAK family, which contain 12 transmembrane regions (Schleyer and Bakker, 1993; Banuelos et al., 1995; Quintero and Blatt, 1997; Fu and Luan, 1998; Kim et al., 1998). All polypeptides from this family have a large hydrophilic loop between the second and third transmembrane spans, an even number of transmembrane domains, and a long, mostly hydrophilic C terminus.

It is well established that the majority of transport proteins function as multimeric proteins. Voltage-gated K^+ channels, for instance, are predicted to consist of six transmembrane regions (S1 to S6) and a pore loop (H5) between domains S5 and S6 (Jan and Jan, 1994). The functioning channel is formed by four identical subunits, and a pore is lined by four H5 loops (Jan and Jan, 1994). Fu and Luan (1998) suggested that the AtKUP1 protein may function as a voltage-dependent K^+ channel. They also found homology between the signature motif (GYGD) in the K^+ channel pore and the IYGD motif in a highly conserved region of the AtKUP1 and HAK1 proteins. We cannot deny the similarity between TRH1 and K^+ channels. In TRH1, however, IYGD is substituted with a modified motif, VFGD. At present, we know neither the number of subunits that form the functional transporter nor whether the VFGD site is in fact a determinant of K^+ selectivity in TRH1. We will attempt to answer these questions in future investigations.

The structural features necessary for K^+ uptake have not been determined for TRH1. However, when the hydrophilic C-terminal end of the protein was removed, the truncated TRH1 polypeptide did not complement the M398 mutant yeast strain, indicating that this domain is essential for potassium translocation. Alternatively, this removal may result in misfolding or misincorporation of the remaining polypeptide in the membrane.

Root Hair Tip Growth

The current paradigm postulates that changes in cell turgor are responsible for the movement of plant cells and organs and for changes in cell shape. A characteristic example of the latter is the opening and closing of stomata, where K^+ and anion channel activities are intimately involved in osmoregulation (Schroeder et al., 1984; Grabov and Blatt, 1998). Although mechanisms similar to those that participate in guard cell volume regulation have been implicated in maize coleoptile elongation (Claussen et al., 1997; Philippar et al., 1999), a role for K^+ translocators in root hair growth has not been demonstrated. It has been shown that a blocker of K^+ channels, tetraethylammonium, inhibits inward-rectifying K^+ channels in Arabidopsis, but it caused only a transient cessation in tip growth (Lew, 1991).

In this report, we demonstrate that the potassium transporter TRH1 is involved in root hair outgrowth. The phenotype of the *trh1* mutant indicates that the identity of the root hair cells is established in the rhizodermis, but root hairs fail to grow despite the fact that other potassium transporters are known to be present in the root. Moreover, the phenotype of the *trh1* mutant plant was not restored even when the plants were grown in a medium containing 50 mM K^+. These results indicate that tip growth requires spatially localized and/or temporally coordinated K^+ transport activity

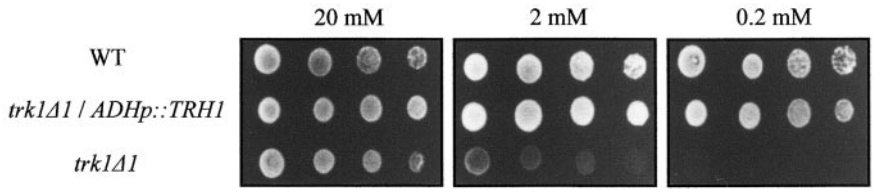

Figure 7. Function of TRH1.

The yeast potassium transport mutant strain M398 lacking the *TRK1* gene was transformed with the yeast expression vector pVT100U containing the *TRH1* gene under the control of the *ADH1* promoter (*trk1Δ1/ADHp*::*TRH1*). The wild-type strain R757 (WT) and the mutant strain M398 (*trk1Δ1*) harbored the plasmid and were used as controls. A fivefold dilution series of cell suspensions were inoculated on ammonium-free solid arginine synthetic medium containing different concentrations of K^+.

that cannot be substituted for by increasing the rate of diffuse K^+ uptake. This specific transport activity may be important for channelized solute delivery or for the generation of electrochemical gradients around tip-growing cells. These gradients have been shown to accompany the apical growth of root hairs as well as some other cells (Jaffe et al., 1974; Schiefelbein et al., 1992; Jones et al., 1995). This hypothesis is compatible with the fact that *trh1* trichoblasts produced multiple initiation sites. More experiments, however, must be performed to prove this theory.

In summary, plants lacking *TRH1* gene function are morphologically normal in most respects under standard growth conditions, indicating either that TRH1 activity is not required for whole-plant development or that other genes can compensate for its absence. TRH1-mediated K^+ translocation is not crucial for overall plant K^+ nutrition, particularly when plants are grown on rich medium. In contrast, there is an absolute requirement for TRH1 activity during root hair elongation, indicating that TRH1 plays a critical role in specific K^+ translocation that is essential for the growth of this cell type.

METHODS

T-DNA Tagging and Plasmid Rescue

Germinating seed from the geographic race Wassilewskija of *Arabidopsis thaliana* were transformed with *Agrobacterium tumefaciens* strain C58C1 Rif^R. This plasmid carries the pGV3850:pAK1003 Ti plasmid (Velten and Schell, 1985; Feldmann, 1991), which contains the neomycin phosphotransferase II gene that confers resistance to kanamycin, and duplicated pBR322 sequences from both ends of the T-DNA. *trh1* plants were identified in a population transformed with this Ti plasmid.

For the recovery of plant DNA disrupted by the T-DNA insertion, the plasmid rescue technique was applied (Behringer and Medford, 1992). The recovered plasmids from both EcoRI-digested (right border rescue) and SalI-digested (left border rescue) genomic DNA isolated from *trh1* homozygous plants were analyzed further.

One plasmid that was rescued from the SalI digest, named pBSalI0.7, consisted of only T-DNA sequences, as if two copies were inserted in a single locus of the genome. Two plasmids were rescued from the EcoRI digest, pBER2.4 and pBER1.3, containing 1.3 and 0.2 kb, respectively, of genomic DNA. T-DNA–plant DNA junction fragments of rescued clones were isolated and either used as hybridization probes or subcloned into pBluescript KS+ or SK− (Stratagene).

Library Screening and Sequencing

The 1.3-kb plant DNA that flanked the T-DNA of the pBER2.4 clone was used as a probe to screen an Arabidopsis genomic library in the λGEM11 vector (Promega). Approximately 100,000 plaques were screened as described by Maniatis et al. (1982). The eight positive overlapping λ clones were subjected to restriction analysis. A 1.5-kb EcoRI genomic fragment was hybridized to a 1.3-kb rescued fragment and identified as being present in all isolated λ clones. This genomic fragment was used as a probe for the screening of a cD4-7 library (Newman et al., 1994). Four positively hybridizing plaques were identified and subjected to plaque purification. The cDNA clones were converted to recombinant pZL1 plasmids. The restriction analysis pattern and sequencing results revealed that all four cDNAs were identical and found to be truncated at the 5′ missing 18 amino acids but homologous with expressed sequence tag (EST) H10G7. Sequencing was performed using a ^{35}S-dATP for double-stranded DNA sequencing (Sanger et al., 1977).

DNA and RNA Gel Blot Analysis

Genomic DNA was isolated from Arabidopsis (Columbia ecotype) by the cetyl-trimethyl-ammonium bromide method (Murray and Thompson, 1980). After digestion with restriction enzymes, DNA (5 μg in each lane) was loaded and separated on a 0.7% agarose gel and blotted onto a nylon membrane (Hybond$^+$; Amersham Corp.). The ^{32}P-labeled probes were prepared using the nick translation protocol. Prehybridization for 1 hr, overnight hybridization, and washes were performed as described by Church and Gilbert (1984).

RNA was isolated from 7-day-old seedlings grown in liquid Murashige and Skoog (1962) (MS) medium. Frozen tissue was ground in liquid nitrogen with a mortar and pestle. Total RNA was isolated using the phenol-SDS extraction method. RNA concentrations were determined spectrophotometrically and verified by ethidium bromide

staining of agarose gels. The RNAs were electrophoresed on 1.2% agarose gels containing formaldehyde and blotted to a nitrocellulose membrane. The membrane was hybridized as described for DNA gel blot analysis.

Polymerase Chain Reaction Analysis

The oligonucleotides used for polymerase chain reaction (PCR) analysis were as follows: st2rev, 5′-CCAAGCTACGCCGCATCTTAC-TCGC-3′, and exon2, 5′-CTTCACCATTGTCATCTGCACTTAAC-3′, corresponding to the 5′ untranslated region and the second exon of the *TRH1* gene, respectively; #1242, 5′-AAGTGCGGCGACGATAGT-CATGCCCCG-3′, corresponding to the pBR322 sequence; apt1, 5′-TCCCAGAATCGCTAAGATTGCC-3′, and apt2, 5′-CCTTTCCCTTAA-GCTCTG-3′, corresponding to the *APT1* gene; actin2, 5′-AAGATG-ACCCAAATCATGTTTGAGAC-3′, and actin3, 5′-ACGACCTTGATC-TTCATGCTGC-3′, corresponding to the *ACTIN* gene. To identify the molecular organization of the T-DNA inserts, PCR reactions were performed using genomic DNA of both wild-type and *trh1* plants as templates. Various combinations of st2rev, exon2, and #1242 were selected. The templates were amplified with the Expand High Fidelity PCR Polymerase (Roche Molecular Biochemicals, Mannheim, Germany). Total RNA (0.5 μg) was isolated and treated with RQ1 RNase-free DNase (Promega). Reverse transcription (RT)–PCR was performed using the Titan one-tube RT-PCR system (Roche Molecular Biochemicals), according to the manufacturer's instructions. The *APT1* gene is expressed at a low level in all tissues of Arabidopsis and was used as an endogenous control mRNA, producing a 478-bp PCR product (Moffatt et al., 1994).

Complementation of the *trh1* Mutation and Medium Preparation

For Arabidopsis transformation, genomic fragments were filled in by Klenow DNA polymerase and inserted into the filled HindIII site of pGPTV-HPT binary vector (Becker et al., 1992). The Agrobacterium strain C58C1 Rif^R containing the nononcogenic Ti plasmid pGV3101 was transformed with the pGPTV-HPT constructs by electroporation (Gene Pulser II; Bio-Rad). The vacuum infiltration method was used for Arabidopsis transformation, according to Bechtold et al. (1993).

Seed (T1 generation) were selected on 1 × MS medium, 2% (w/v) sucrose, 0.05% Mes, pH 5.7, 0.6% (w/v) agarose plates containing 30 mg/L hygromycin (Sigma) and 50 mg/L Claforan (Hoechst, Frankfurt, Germany). Resistant individuals could be clearly identified 10 to 15 days after germination. Seed of T2 and T3 generations were plated individually on the medium described above, except that Claforan was omitted.

To resolve the effects of various concentrations of K^+ on root hair morphogenesis in both wild-type and *trh1* plants, artificial plant growth medium was prepared according to Hirsch et al. (1998). The primary roots of 7-day-old seedlings were observed and photographed using an Olympus (Tokyo, Japan) SZX12 stereomicroscope.

^{86}Rb Uptake Experiments

Plants were grown hydroponically for 7 days in sterile conditions on 1 × MS medium, 1% sucrose, and 0.05% Mes, pH 5.7. Seedlings were harvested and preincubated for 5 hr in medium containing 10 μM KNO_3, 3 mM $Ca(NO_3)_2$, 1.5 mM $MgSO_4$, 1 mM NaH_2PO_4/Na_2HPO_4 buffer, pH 5.6, and 7 mM Ca^{2+}-Mes buffer, pH 5.6. Incubation buffer of the same composition was supplemented with 1.8 μM ^{86}Rb-Cl (DuPont–New England Nuclear, Boston, MA) with a specific activity of 21.9 Bq/pmol. After loading with ^{86}Rb, samples (three seedlings in each) were washed with 10 × 5 mL of preincubation buffer supplemented with 50 μM unlabeled Rb. Samples were dried for 15 sec on 55-mm Ø paper filters (Whatman) using a vacuum filtration apparatus and placed in preweighed scintillation vials. After determination of plant weight, vials were filled with Ecolite (ICN, Costa Mesa, CA) scintillation cocktail. Radioactivity was measured using a Rackbeta 1211 (LKB-Wallac, Turku, Finland) scintillation counter.

Yeast Complementation

To determine whether TRH1 can function as a potassium transporter, we tested cDNA for the ability to complement the potassium transport deficiency of the M398 strain of *Saccharomyces cerevisiae* (Gaber et al., 1988; Sentenac et al., 1992). The H10G7 EST full-length cDNA (GenBank accession number AA042476) was cloned into the XbaI site of the yeast expression vector pVT100U that carries the constitutively expressed system of the yeast alcohol dehydrogenase gene (*ADH1*) promoter–terminator sequences and a modified *URA3* gene for selection in yeast (Vernet et al., 1987). Yeast transformation was performed by the lithium acetate method. The transformed yeast cells were selected on URA-glucose-ammonium–free solid arginine synthetic medium supplemented with 100 mM KCl (Rodriguez-Navarro and Ramos, 1984; Ko et al., 1990). Growth restoration of the M398 mutant strain was performed on the synthetic medium described above supplemented with various concentrations of K^+.

Microscopy

Three- and five-day old roots were frozen in nitrogen slush at −190°C. Ice was sublimed at −90°C, and the specimen was sputter coated and examined on an XL 30 FEG (Philips, Eindhoven, The Netherlands) cryoscanning electron microscope fitted with a cold stage.

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