The TMK Subfamily of Receptor-Like Kinases in Arabidopsis Display an Essential Role in Growth and a Reduced Sensitivity to Auxin

Ning Dai1a, Wuyi Wang1b, Sara E. Patterson2a, Anthony B. Bleecker1†

1 Department of Botany and Laboratory of Genetics, University of Wisconsin-Madison, Madison, Wisconsin, United States of America, 2 Department of Horticulture, University of Wisconsin-Madison, Madison, Wisconsin, United States of America

Abstract

Mechanisms that govern the size of plant organs are not well understood but believed to involve both sensing and signaling at the cellular level. We have isolated loss-of-function mutations in the four genes comprising the transmembrane kinase TMK subfamily of receptor-like kinases (RLKs) in Arabidopsis. These TMKs have an extracellular leucine-rich-repeat motif, a single transmembrane region, and a cytoplasmic kinase domain. While single mutants do not display discernible phenotypes, unique double and triple mutant combinations result in a severe reduction in organ size and a substantial retardation in growth. The quadruple mutant displays even greater severity of all phenotypes and is infertile. The kinematic studies of root, hypocotyl, and stamen filament growth reveal that the TMKs specifically control cell expansion. In leaves, TMKs control both cell expansion and cell proliferation. In addition, in the tmk double mutants, roots and hypocotyls show reduced sensitivity to applied auxin, lateral root induction and activation of the auxin response reporter DR5: GUS. Thus, taken together with the structural and biochemical evidence, TMKs appear to orchestrate plant growth by regulation of both cell expansion and cell proliferation, and as a component of auxin signaling.

Introduction

During development, it is essential for all multicellular organisms to perceive and process information from extracellular signals via cell surface receptors to control organ size and growth. In animals, the evolutionarily conserved receptor tyrosine kinase (RTK) signaling pathway plays a central role in regulating cell proliferation, cell growth, cell differentiation and survival [1], [2]. RTKs comprise a large family of integral membrane proteins with highly divergent extracellular domains coupled to a conserved intracellular tyrosine kinase motif. Plant receptor-like kinases (RLKs) exhibit a basic structural similarity with animal RTKs, but also display several unique characteristics. Most plant RLKs are serine/threonine kinases and have extracellular domains distinct from ligand-binding motifs of RTKs in animals [3], [4]. Structural features of plant RLKs support the idea that they may also function as entry points for signaling pathways.

In Arabidopsis, there are more than 400 RLKs with divergent extracellular motifs and cytoplasmic serine/threonine kinase domains [3]. While examples are still limited, several plant RLKs have been assigned functions including brassinosteroid signaling (BRI1) [5–6], meristem development (GLV1) [7], floral organ abscission (HAESA, HAESA-LIKE2, and EVERSHEDE) [8–10], pollen tube and ovule development (FERONIA) [11], cell wall signaling such as lignin deposition, cell expansion, and cell elongation (FERONIA; THESEUS 1; HERCULES 1 and 2) [12–16], drought and salt stress responses [17] and inflorescence development [18]. Ligands have been identified for BRI1 [19], HAESA [9], the systemin and phytosulfokine receptors [20], the ERECTA-family RLKs [21] and the CLV1/BAM family [22]. It has also been shown that closely related RLKs can bind ligands with similar functions and yet that recognition mechanisms are quite complex.

TMK1 encodes a RLK with leucine-rich repeats in the extracellular domain and was previously found to be expressed in all major organs [23]. This early work also demonstrated autophosphorylation of the kinase domain on serine and threonine residues with a preference for Mn2+ and Mg2+ [24]. Despite these molecular and biochemical analyses of TMK1, its biological function was not determined. The TMK1 loss-of-function mutant does not show any growth or developmental abnormality compared to wild type [25], suggesting that other RLKs in Arabidopsis are capable of compensating for the function of TMK1. In this study, we explore the biological function of all four members in the TMK subfamily of RLKs in Arabidopsis and present evidence that the four members of the TMK subfamily...
play a critical role in the control of cell expansion and cell proliferation. Mutant combinations among the TMKs result in plants that are smaller in size as a consequence of reduced cell size in roots, hypocotyls and stamen filaments, and decreased cell size and cell number in leaves. Hormone assays demonstrated that mutant combinations have reduced sensitivity to auxin in lateral root induction, reduced DR5:GUS activation, and that the tmk1;tmk3;tmk4 triple mutant is insensitive to applied auxin. Our study indicates that the four members of the TMK subfamily act in a functionally redundant manner to mediate both cell expansion and proliferation downstream of auxin.

Results

The TMK Subfamily Members Show Overlapping Patterns of Tissue Expression

Phylogenetic analysis of all RLKs from Arabidopsis based on kinase domain sequence alignment revealed a distinct TMK subfamily composed of TMK1 and three other RLKs, designated TMK2, TMK3, and TMK4 (Fig. 1A). Several orthologous sequences are present in rice (Os) [26], tobacco (Nt) [27] and soybean (Glycine max) [28], indicating potential roles for the TMK subfamily in both dicot and monocot species (Fig. 1A). The four members of the Arabidopsis TMK subfamily possess a similar domain organization, containing multiple LRR repeats in the extracellular region with a characteristic intervening sequence separating LRRs into two blocks. In addition, members of the TMK subfamily contain a unique intron in the same location in the kinase domain (Fig. 1B).

To determine expression patterns of the members of the TMK subfamily, a semiquantitative RT-PCR analysis was performed. TMK1, TMK3 and TMK4 were expressed at comparable levels in all organs tested, including roots, stems, leaves, flowers and siliques (Fig. 1C). On the other hand, TMK2 was detected in trace amounts only in flowers and siliques (Fig. 1C). The expression patterns of the TMK subfamily members were further confirmed by analysis of the β-glucuronidase (GUS) reporter expressed under the control of their native promoters (Fig. 1D). All four TMKs were expressed in similar tissues in seedlings and flowers; however, TMK 2 expression was significantly lower and only detected when seedlings are exposed in the staining solution for an extended period of time.

TMK1 Localizes to the Cellular Membranes

Based on the protein sequences, the four members in the TMK subfamily are predicted to be transmembrane proteins. In order to evaluate this prediction, the entire TMK1 coding region was translationally fused with modified green fluorescent protein (GFP) in frame at the C-terminus. This fusion construct TMK1:GFP, driven by the native TMK1 promoter, complements the tmk1;tmk4 mutant phenotype (Table 1). Confocal microscopic analysis of TMK1:GFP transgenic plants revealed that TMK1 localizes to the cell periphery (Fig. 1E). Western blotting analysis further confirmed TMK1 membrane localization; as shown in Figure 1F in which TMK1 was highly enriched in the pellet fraction containing cellular membranes. TMK1 was not detectable in the soluble fraction.

Combinatorial Loss of Function of TMKs in Arabidopsis Markedly Inhibits Growth and Development

The homozygous tmk1, tmk2, tmk3 and tmk4 mutants containing a T-DNA insertion in the coding region of the genes were identified and verified by PCR and sequencing (Fig. 1B). We could not detect the full-length cDNA of any of the corresponding TMKs in the T-DNA insertion lines supporting the selection of homozygous lines for loss of function. All mutants were backcrossed five generations and then carefully observed throughout their entire life cycle, and no significant differences in growth or development relative to wild type were observed in any of the single mutant lines (Table 1). To assess functional redundancy, we constructed all possible double and triple mutants, as well as the quadruple mutant. While most double mutants did not display any significant altered phenotypes (Table 1), the tmk1;tmk4 double mutant displayed a severe reduction in organ size, a substantial retardation in growth, a delay in development, and a decrease in fertility (Fig. 2; Table 1 and Table 2). The tmk1;tmk3;tmk4 triple mutant showed increased severity of all phenotypes observed in tmk1;tmk4 double mutants, while the quadruple mutant was comparable to the triple mutant in vegetative growth (Table 1); however, completely infertile (Fig. 2H and 2K; and Table 1).

The seedlings of all mutants appeared normal at germination; however, after four days of growth on vertical agar plates, the root length of the tmk1;tmk4 double mutant was about 1/3 that of wild type and the root length of the tmk1;tmk3;tmk4 triple mutant was further reduced to approximately 1/6 that of wild type (Fig. 2A and Table 1). Hypocotyls of tmk1;tmk4 and tmk1;tmk3;tmk4 etiolated seedlings were three and five times shorter than those of wild type, respectively (Fig. 2B and Table 1). Vegetative shoot development was also retarded as indicated by a chronological delay in flowering time (Table 2 and Fig. 2C–E). Both the tmk1;tmk4 double and tmk1;tmk3;tmk4 triple mutants produced the same number of rosette leaves as wild type at bolting, but required a significantly longer time to bolt (Table 2). Rosette diameters of the tmk1;tmk4 double and the tmk1;tmk3;tmk4 triple mutants are about half and one third of wild type, respectively (Table 1 and Fig. 2C–E).

In contrast to leaf, hypocotyl and root development, the inflorescence shoot of all mutant combinations was similar to wild type in terms of internode length and flower size (Fig. 2F–G and Table 2). However, the initiation of bolting was delayed, and the height of the primary inflorescence and the length of stamen filaments were significantly reduced (Table 2 and Fig. 2G). Approximately 1/4 the number of flowers were produced on the primary inflorescence of the quadruple mutant, and the mutant was completely infertile (Table 2 and Fig. 2K). Reductions in fertility were also observed in the double tmk1;tmk4 and the triple mutant tmk1;tmk3;tmk4 (Fig. 2 H–J). In order to further understand the lack of fertility in tmk1 mutant combinations, we tried to restore fertility by pollinating with both mutant and wild-type pollen. We found that pollen from the respective mutant parent lines restored fertility in tmk1;tmk4 and tmk1;tmk3;tmk4, but not the quadruple mutant. Pollination with wild-type pollen also restored fertility in the double and triple mutants yet only partially in the quadruple.

To confirm that the phenotypes of the mutants are caused by the loss of function of TMKs, genomic clones of each gene were transformed into mutant backgrounds. Growth of tmk1;tmk4 was restored to wild type by either the TMK1 or TMK4 genomic clone (Table 1 and Fig. 2L–O). Similarly, transformation of tmk1;tmk3;tmk4 with the TMK3 genomic clone resulted in partial restoration of the triple mutant phenotype to one similar to, or less severe than, that of the tmk1; tmk4 double mutant (Table 1). Consistent with the expression pattern of TMK2, the genomic clone of TMK2 partially rescued the infertility of the quadruple mutant (Table 1). As indicated, we obtained complete restoration of fertility with either TMK1 or TMK4 alone in the double mutant tmk1;tmk4. The partial rescue of tmk1;tmk3;tmk4 and the quadruple tmk1;tmk2;tmk3;tmk4 by TMK2 indicates functional redundancy between all
Figure 1. TMK Subfamily of RLKs and Gene Expression Patterns. (A) The phylogenetic tree of the TMK subfamily and its closest relatives in flowering plants. TMK1 (At1g66150); TMK2 (At1g24650); TMK3 (At2g01820) and TMK4 (At3g23750) are all from Arabidopsis. OsTMK, Os9632 and Os 9639 are from Oryza sativa, NtTMK1 from Nicotiana tabacum, and RHG4 from Glycine max. (B) Domain organizations showing intron locations (green triangle) and T-DNA positions (red triangle) for each of the TMK family members. Shared features include signal peptide (yellow), multiple leucine rich repeats (green), transmembrane region (black) and kinase domain (blue). (C) Quantitation of transcript abundance for each member of the TMK subfamily of RLKs. TMK1, TMK3 and TMK4 are expressed at approximately equal levels in all organs examined, while TMK2 expression can only be detected in the flowers and siliques. (D) Expression patterns of GUS reporter driven by TMK1, TMK2, TMK3 and TMK4 native promoters in seedlings and flowers. Scale bars: 10 mm. (E) Expression of TMK1 is associated with the plasma membrane in the mature zone of root in Arabidopsis as indicated by TMK1:GFP lines in which the TMK1 promoter was translationally fused with GFP. Scale bar: 50 μm. (F) TMK1 is an integral membrane protein as determined by crude protein extracted from Arabidopsis seedlings. Note expression of other membrane-associated proteins AHA2 and SEC12 as controls. T: total protein, P: membrane pellet, S: soluble fraction.
doi:10.1371/journal.pone.0060990.g001
members of the family. In summary, the complete loss of function of TMKs in Arabidopsis markedly inhibits growth and development of most organs, including roots, hypocotyls, leaves, and stamen filaments.

**Cellular Defects of tmk Mutants**

We hypothesized that the reduced organ size in the mutants could be due either to a reduction in cell size, cell number or both. To investigate the cellular basis of the tmk1; tmk4 double mutant, tmk1; tmk3; tmk4 triple mutant and quadruple mutant phenotypes, we measured cell size and cell number in roots, hypocotyls, leaves, and stamen filaments.

In the root, the length of mature cortical cells in the tmk1; tmk4 double mutants, and tmk1; tmk3; tmk4 triple mutants is approximately 1/3 and 1/4 that of wild type, respectively (Fig. 3A). However, the number of cortical cells is similar in the mutants and wild type (Table 3). This result was supported by analysis of the cell division marker Cyc1At:GUS [29], which demonstrated that wild type (Table 3). These observations clearly suggest that TMKs contribute to the regulation of cell expansion in roots, hypocotyls, and stamen filaments.

While these observations clearly indicate a role of TMKs in the regulation of cell expansion, we were also interested in determining if rates of cell division were also affected by loss of TMKs. A kinematic analysis of the root growth was performed to gain a deeper understanding of the cellular basis of the short root phenotype of the tmk1; tmk4 and tmk1; tmk3; tmk4 mutants. Rates of cell expansion, cell division, and the duration of time that a cell spent in the root elongation zone were calculated [30]. The results show that the effects of the tmk mutations on root growth could be attributed to a reduction in both the rate and duration of cell expansion (Table 4). In contrast to the finding that the decreased length of tmk mutant roots, hypocotyls and stamen filaments is due to a reduction in cell expansion, the decrease in leaf size was primarily due to a reduction in cell number. For example, measurements of mature epidermal pavement cells of tmk1; tmk4 (Fig. 4A–C), we hypothesized that cell proliferation is also affected in the mutant leaves. We observed that patterning of cells in the epidermis and overall structure of pavement, guard, and trichome cells were primarily wild type in the mutants. Rates of cell division and the duration of time that a cell spent in the root elongation zone were also calculated [30]. The results show that the effects of the tmk mutations on root growth could be attributed to a reduction in both the rate and duration of cell expansion (Table 4).

### Table 1. Phenotypic characterization of tmk mutants.

| Genotype                  | Root Length 4DAG (mm) | Etiolated Hypocotyl Length 4DAG (mm) | Maximum Rosette Diameter (cm) | Seeds/Silique |
|---------------------------|-----------------------|--------------------------------------|-------------------------------|--------------|
| Wild type                 | 21.8 ± 1.4            | 10.6 ± 1.1                           | 7.5 ± 0.9                     | 50.2 ± 7.1   |
| tmk1                      | 22.5 ± 1.9            | 10.2 ± 1.5                           | 7.2 ± 1.0                     | 48.6 ± 6.5   |
| tmk2                      | 21.6 ± 1.3            | 10.0 ± 1.7                           | 6.9 ± 0.7                     | 48.9 ± 9.2   |
| tmk3                      | 23.4 ± 1.7            | 11.2 ± 1.8                           | 8.1 ± 1.1                     | 51.2 ± 6.8   |
| tmk4                      | 24.8 ± 1.5            | 10.5 ± 1.4                           | 7.4 ± 0.8                     | 46.3 ± 8.1   |
| tmk1;tmk2                 | 21.2 ± 1.2            | 10.2 ± 1.7                           | 7.9 ± 0.9                     | 47.2 ± 9.3   |
| tmk1;tmk3                 | 22.6 ± 1.5            | 10.9 ± 1.6                           | 7.1 ± 0.8                     | 42.8 ± 8.7   |
| tmk1;tmk4                 | 6.9 ± 0.9*            | 4.2 ± 0.8*                           | 3.6 ± 0.5*                    | 15.4 ± 3.6*  |
| tmk2;tmk3                 | 20.4 ± 1.5            | 10.7 ± 1.5                           | 7.2 ± 0.7                     | 44.3 ± 7.5   |
| tmk2;tmk4                 | 22.1 ± 1.3            | 10.3 ± 1.7                           | 7.8 ± 0.6                     | 48.6 ± 6.7   |
| tmk3;tmk4                 | 21.4 ± 1.4            | 10.8 ± 1.3                           | 7.5 ± 0.8                     | 45.6 ± 7.8   |
| tmk1;tmk2;tmk4            | 6.6 ± 0.7*            | 4.4 ± 0.7*                           | 3.1 ± 0.4*                    | 14.6 ± 3.4*  |
| tmk1;tmk2;tmk3            | 21.4 ± 1.1            | 10.0 ± 0.8                           | 7.6 ± 0.6                     | 49.4 ± 7.1   |
| tmk1;tmk3;tmk4            | 3.4 ± 0.4*            | 2.6 ± 0.7*                           | 2.1 ± 0.3*                    | 4.6 ± 1.3*   |
| tmk2;tmk3;tmk4            | 19.6 ± 0.8            | 9.9 ± 1.7                            | 6.9 ± 0.7                     | 50.1 ± 8.3   |
| tmk1;tmk2;tmk2;tmk4       | 3.2 ± 0.3*            | 2.4 ± 0.6*                           | 2.1 ± 0.4*                    | 0*           |
| tmk1;tmk2;tmk3;tmk4       | 21.8 ± 1.7            | 9.9 ± 1.0                            | 7.6 ± 1.0                     | 42.7 ± 9.1   |
| tmk1;tmk4;gTMK1           | 22.2 ± 1.9            | 10.2 ± 1.1                           | 7.7 ± 1.1                     | 41.7 ± 11.2  |
| tmk1;tmk3;tmk4;gTMK3      | 6.3 ± 0.6*            | 4.2 ± 0.8*                           | 3.7 ± 0.9*                    | 11.4 ± 5.4*  |
| tmk1;tmk2;tmk1;tmk4;gTMK2 | 3.4 ± 0.5*            | 2.8 ± 0.9*                           | 2.3 ± 0.4*                    | 3.8 ± 2.1*   |
| tmk1;tmk3;gTMK1-GFP       | 20.9 ± 1.6            | 10.4 ± 1.2                           | 7.5 ± 0.9                     | 46.0 ± 6.0   |

Wild-type plants were compared with various tmk mutant combinations and transgenic plants. The average ± standard deviation is shown for each measurement (n>30). Values that are significantly different (p<0.001) from wild type are labeled with an asterisk. 4 DAG: 4 days after germination.

doi:10.1371/journal.pone.0060990.t001
Thus, the decreased leaf size of tmk1; tmk4 is due in large part to reduced cell proliferation during early leaf development.

In the process of cell division, cell proliferation is often accompanied by cell growth, except in the early stages of embryogenesis in animals [31]. An intracellular mechanism monitors cell growth and halts progression of cell proliferation through the cell cycle at specific G1 or G2 checkpoints if the cell has not grown to a threshold size [32]. We hypothesized that the reduced frequency of cell proliferation in the leaf primordia of
**Table 2. Phenotypic characterization of tmk mutants.**

| Genotype             | Days to Bolting | # of Rosette Leaves | Internode Length (cm) | # of Flowers/Primary Inflorescence | Maximum Height/Primary Inflorescence (cm) |
|----------------------|-----------------|---------------------|-----------------------|-----------------------------------|------------------------------------------|
| Wild type            | 26.8±3.9        | 11.2±1.1            | 0.72±0.18             | 45.8±4.9                          | 40.8±5.7                                 |
| tmk1;tmk4            | 42.8±4.6*       | 11.8±1.3            | 0.73±0.13             | 18.9±3.6*                         | 20.6±3.2*                                |
| tmk1;tmk3;tmk4       | 49.9±3.8*       | 10.9±1.4            | 0.69±0.16             | 15.7±3.5*                         | 12.1±2.9*                                |
| tmk1;tmk2;tmk3;tmk4  | 49.1±4.1*       | 11.0±1.4            | 0.70±0.11             | 11.4±3.1*                         | 9.8±2.1*                                 |

Wild-type plants were compared with tmk mutants. The average ± standard deviation is shown for each measurement (n=20). Values that are significantly different (p<0.01) from wild type are labeled with an asterisk.

doi:10.1371/journal.pone.0060990.t002

**Altered Responses of TMK Mutants to the Growth Regulator Auxin**

Due to our observations on reduction in cell elongation and cell division, we screened tmk seedlings for altered responses to growth regulators: abscisic acid (ABA), auxin, cytokinins, ethylene, and gibberellins. Whereas mutant seedlings responded relatively normally to ethylene, ABA and cytokinin (data not shown), the double mutant tmk1; tmk4 showed reduced sensitivity to applied auxin and the triple mutant tmk1; tmk3; tmk4 was insensitive to applied IAA (Fig. 5A). Similar results were obtained for etiolated hypocotyls (Fig. 5B). Roots of the mutant also showed a significant reduction in auxin stimulated lateral root development (Fig. 5C). Further evidence for altered auxin sensitivity was obtained by crossing the auxin responsive DR5::GUS reporter gene into the tmk1; tmk4 background. Whereas the root tip of tmk1; tmk4 mutant seedlings showed a normal DR5::GUS expression pattern without applied auxin, the elongation zone and part of mature zone of the root showed a greatly reduced response to applied IAA, 2,4-D and NAA (Fig. 5D). This suggests that auxin sensitivity is affected early in the auxin signaling pathway, given that DR5 is a reporter for the activity of AUX/IAA transcriptional factors [34].

**Discussion**

We observed that the increasing loss of members of the TMK subfamily of RLKs in Arabidopsis leads to increased severity of a suite of phenotypes without revealing phenotypic effects specific to individual isoforms. The lack of a detectable phenotype in any double or triple mutant combination that leaves a functional TMK1 or TMK4 intact indicates that either of these genes is
Table 3. Cellular characterization of roots, hypocotyls and stamen filaments of tmk mutants.

| Genotype     | Root (3 days after germination) | Etiolated Hypocotyl (4 days after germination) | Stamen Filament (at anthesis) |
|--------------|--------------------------------|-----------------------------------------------|-------------------------------|
|              | Length (mm) | Cortical Cell Length (µm) | Cortical Cells/ File | Length (mm) | Cortical Cell Length (µm) | Cortical Cells/ File | Length (mm) | Epidermal Cell Length (µm) | Epidermal Cells/ File |
| Wild type    | 12.9±1.6    | 179.2±9.8               | 48.4±1.5          | 10.6±1.1   | 1.6±0.5                | 22.3±0.8          | 3.1±0.3     | 134±24                        | 17.6±2.4              |
| tmk1;tmk4    | 4.3±0.7*    | 37.6±8.9*               | 49.1±1.8          | 4.2±0.8*   | 0.7±0.2*               | 22.1±0.7          | 2.4±0.2     | 109±26*                        | 17.2±2.1              |
| tmk1;tmk2;tmk4| 3.7±0.4*   | 28.3±6.4*               | 47.9±2.1          | 2.6±0.7*   | 0.6±0.2*               | 21.7±1.1          | 1.9±0.2     | 93±25*                         | 17.6±2.5              |
| tmk1;tmk2;tmk4| 3.6±0.4*   | 27.4±7.2*               | 47.7±2.3          | 2.4±0.6*   | 0.5±0.2*               | 21.9±0.9          | 1.7±0.1*    | 89±22*                         | 17.5±2.1              |

Wild-type plants were compared with tmk mutants at the same age (n = 20). Cells were measured for each tissue (n = 100). The average ± standard deviation is shown. Values that are significantly different (p < 0.01) from wild type are labeled with an asterisk.

doi:10.1371/journal.pone.0060990.t003

Table 4. Analysis of duration and rate of average root growth in tmk mutants.

| Genotype     | Cell production rate (cells/hour) | Cell residence time (hours) | Average growth rate (1/h) |
|--------------|----------------------------------|----------------------------|---------------------------|
| Wild type    | 0.97±0.2                         | 25.1±4.1                   | 0.165±0.004               |
| tmk1;tmk4    | 0.94±0.3                         | 14.9±3.6*                  | 0.113±0.008*              |
| tmk1;tmk2;tmk4| 0.98±0.3                        | 10.4±3.3*                  | 0.119±0.009*              |

Wild-type plants were compared with tmk1;tmk4 and tmk1;tmk2;tmk4 mutants (n = 20). Cell size and cell number from the cortical cell file along different zones of roots were measured for each genotype. Numbers indicate the average ± standard deviation. The average growth rate = (ln(final cell size) – ln(initial cell size))/residence time. Values that are significantly different (p < 0.01) from wild type are labeled with an asterisk.

doi:10.1371/journal.pone.0060990.t004
Table 5. Analysis of cell division and cell size in tmk mutant leaves.

| Genotype        | Size of the 6th leaf (cm²) | Size of adaxial mature epidermal pavement cells (μm²) | Frequency of cells showing Cyc1At:GUS activity |
|-----------------|----------------------------|------------------------------------------------------|----------------------------------------------|
| Wild type       | 3.04 ± 0.28                | 1431 ± 412                                           | 4.3% ± 0.6%                                  |
| tmk1;tmk4       | 0.28 ± 0.08*               | 1212 ± 376*                                          | 1.2% ± 0.4%*                                 |
| tmk1;tmk3;tmk4  | 0.15 ± 0.04*               | 1132 ± 382*                                          | ND                                            |
| tmk1;tmk2;tmk3;tmk4 | 0.16 ± 0.03*           | 1118 ± 394*                                          | ND                                            |

Wild-type plants were compared with various tmk mutant combinations. Adaxial epidermal pavement cells were measured in 20 plants for each genotype. The average ± standard deviation is shown. Values that are significantly different (p < 0.01) from wild type are labeled with an asterisk. The frequency of cells showing Cyc1At:GUS was determined by number of adaxial epidermal pavement cells stained with Cyc1AtGUS/total number of adaxial epidermal pavement cells. ND: not determined.

doi:10.1371/journal.pone.0060990.t005

**tmk Mutants Showed Reduced Response to Applied Auxin**

The growth defects of the tmk1;tmk4 double and tmk1;tmk3;tmk4 triple mutants indicate that the TMK genes are required for normal plant growth. While the specific growth phenotypes of these mutants do not fit previously described profiles for mutations in known hormone pathways, it is possible that TMKs are involved in novel aspects of hormone pathways. The insensitivity of the tmk1;tmk3;tmk4 triple mutant roots and hypocotyls to applied auxin is a stronger phenotype than most existing auxin-resistant mutants, and this reduced sensitivity to applied auxin was also indicated by the suppression of auxin-induced lateral root development and reduction in induction of DR5:GUS expression in the higher order tmk mutants. The loss of TMK activity appears to specifically affect auxin signal transduction in root and potentially shoot development. While the role for auxin in cell expansion is well established, understanding auxin’s role in organ development is less well characterized. Further investigation of growth factor(s) and other components involved in the TMKs signal transduction pathway will help us understand plant organ size control at both molecular and cellular levels.

**Methods**

**Phylogenetic Analysis**

The protein sequence of the TMK1 kinase domain was used to conduct BLAST searches against sequences deposited in the GenBank database. The most closely related proteins with an E value cutoff of 1 x 10^-20 and the well-studied Arabidopsis RLKs, CLAVATA1, BRI1 and ERECTA were further used for sequence alignment and phylogenetic analysis. The kinase domain sequences were aligned using MacClade and the phylogenetic tree was generated with PAUP using the neighbor-joining method. The bootstrap values are shown on the nodes [4].

**Cell Fractionation and Immunoblot Analysis**

Arabidopsis ecotype Columbia (COL) wild-type seeds were germinated and grown in MS liquid culture. Tissues were harvested after 10 days and homogenized with extraction buffer (20 mM Tris, pH 8.5, 150 mM NaCl, 1 mM EDTA, 20% glycerol, and 1 mM PMSF, 1 unit/mL macroglobulin, 1 μg/mL pepstatin, 10 μg/mL aprotinin, and 10 μg/mL leupeptin as protease inhibitors). The homogenate was strained through Miracloth and centrifuged at 100,000 g for 30 min. The pellet membrane fraction was resuspended in solubilization buffer (10 mM Tris, pH 7.3, 150 mM NaCl, 1 mM EDTA, 10% glycerol, 1% Triton X-100) containing the protease inhibitors listed above, and centrifuged again at 100,000 g for 30 min. Samples were subjected to SDS-PAGE and subsequent Western blot analysis using α-CT antiserum as described [23].

**Growth Conditions**

Mutants and wild-type Arabidopsis seeds were surface-sterilized in 10% chlorox for 10 minutes and rinsed with sterile water or 70% (v/v) ethanol for 2 min, dried on sterile filter paper, and placed on 1/2 MSNS (half-strength Murashige and Skoog salts without sucrose) agar plates, or directly sown in soil. Seeds were stratified at 4°C for 2 days in the dark prior to being transferred to a growth chamber (Econair Ecological Chamber, Winnipeg, Canada) set at 22°C in a 16-h light (125 μE m^-2 s^-1) and 8-h dark regime. About 7 days after germination, seedlings were transferred to soil, a mixture (2:1, v/v) of Jiffy Mix (Jiffy products of America, Batavia, IL) and Perlite (Midwest Perlite, Appleton WI), and grown until the plants were photographed or reached maturity.

**Mutant Isolation**

The T-DNA insertion lines tmk3 and tmk4 for TMK3 (At2g1820) and TMK4 (At3g23750), respectively, were isolated following the instructions of the Wisconsin knock-out facility. The tmk1 mutant for TMK1 (At1g66150) was identified in the T-DNA Express Collection at the Salk Institute (Salk_016360) [50], and the tmk2 mutant for TMK2 (At1g24650) was isolated from the Syngenta collection (Garlic_1242) [51]. All mutant lines were backcrossed five times to COL wild type prior to generation of double, triple, and quadruple mutants for any phenotypic analysis. The isogenic wild type COL was used as control in all phenotypic analyses except when specified as LER. LER lines were generated after selection of mutant lines in COL ecotype and backcrossed five times to LER wild type prior to generation of the double mutant tmk1;tmk4. The following gene-specific primers were used for genotyping:

- **TMK1** (At1g66150), 5’-CGATCCTTTGTAACACTG-GATTGGGATA-3’ and 5’-CCGAAACT-TAGAGTGATTGTTT-GTGGTTA-3’
- **TMK2** (At2g1820), 5’-CCGAAATC-AGTATTTCGTTGC-GTTCCTACTGAA-3’ and 5’-GAAGA GTGGACCGA-TTCTCCATTGCTT-3’
- **TMK3** (At3g23750), 5’-GACCTAGGAATGTCAGGAACGATCGAA-3’ and 5’-TGACCTCCAATGCAGAGAATGTCAGGA-3’
- **TMK4** (At1g24650), 5’-TTCCACCTGACCGGAACTTAAGGT-3’

Pesticide conditions are: 95°C for 2 min; 95°C 20 s/60°C 20 s/72°C 30 repeated for 34 cycles; 72°C for 10 min.
Semi-quantitative Reverse Transcriptase-mediated Polymerase Chain Reaction (RT-PCR)

Isolation of total RNA, cDNA synthesis, and RT-PCR were carried out as described previously described by Bleecker lab [52,53]. The TMK1, TMK2, TMK3, and TMK4 PCR products were amplified using gene specific primers, detected by transillumination, photographed with a Kodak digital camera, and quantified with the ImageQuant software (Molecular Dynamics, Sunnyvale, CA). Data analysis was performed as described previously [54]. The following gene-specific primer pairs for each member of the TMK subfamily were used for RT-PCR: TMK1 (At1g66150), 5'-CACCAGAGCTTTATACACAGGGATCTT-3' and 5'-TCGCTTTCTTGAATGATGCT-3'; TMK2 (At1g24650), 5'-CACAATCAATTGAGACCAAGATT-3' and 5'-ATCTCGCGGTTCCCTGGAGCTACATTGATT-3'; TMK3 (At2g01820), 5'-AAGGGCGAAAACCGTTAGAC-3' and 5'-GCATTTTCGTCTTTGCTGGCTGCT-3', and TMK4 (At3g23750), 5'-ACTCGGTTACTCTCCTTTGACA-3' and 5'-TGAGAACCACACACAAATGCA-3'.

Plasmid Construction and Plant Transformation

The genomic fragments of TMK1 (6055 bp), TMK2 (5675 bp), TMK3 (6033 bp), and TMK4 (5663 bp) were amplified using PCR and cloned into pPZP221 [55] pPZP221B, a derivative of

Figure 4. TMKs Regulate Cell Proliferation During Leaf Development. (A) Fully expanded leaf 6 of wild type (left) and tmk1; tmk4 double mutant (right). (B) Cell size of leaf epidermal pavement cells in wild type. (C) Cell size of leaf epidermal pavement cells in the tmk1; tmk4 double mutant. (D) The frequency of cell division (cells stained with blue/total cells) in the primordia of leaf 6 in wild type. (E) The frequency of cell division (cells stained with blue/total cells) in the primordia of leaf 6 in the tmk1; tmk4 double mutant. (F) Expansion rate of leaf 6. Leaf emergence was determined, as seedlings emerged with minimum leaf length greater than 2 mm. (G) The size of dividing adaxial epidermal pavement cells (stained with blue) in the primordia of leaf 6 in wild type and in the tmk1; tmk4 double mutant. Scale bars: (A) = 5 mm; (B)-(C) = 10 μm; (D)-(E) = 20 μm. doi:10.1371/journal.pone.0060990.g004
pPZP211 that was generously provided by Sebastian Bednarek (University of Wisconsin, Madison) and in which the aacCl gene (gentamycin selectable marker) is replaced with the bar gene (glufosinate/bialphos selectable marker) [56]. These genomic clones contain the 5′ flanking region upstream of the start codon and the 3′ flanking region downstream of the stop codon with lengths of 1946 and 1196 bp (TMK1), 1977 and 952 bp (TMK2), 2011 and 1004 bp (TMK3), and 1862 and 869 bp (TMK4), respectively. These assignments of nucleotide positions were based on the annotation in the TAIR database.

To generate the β-glucuronidase (GUS) fusion constructs (TMK:GUS), a SmaI to EcoRI fragment consisting of the GUS gene and the NOS terminator from pBI121 was inserted into pPZP211. The fragments of TMK1, TMK2, TMK3, and TMK4 consisting of the 5′ flanking regions and the first few N-terminal amino acids were then ligated in frame into pPZP211 upstream of GUS.

For the TMK1:GFP fusion construct, a 370-bp NOS terminator was amplified from the plasmid pCD64 kindly provided by Chris Day (University of Wisconsin, Madison) and inserted into pPZP221B at restriction sites PstI and HindIII. An EcoRI to SpeI TMK1 fragment consisting of the 5′ flanking region and the complete genomic coding region of the genomic TMK1 clone and an XbaI to PstI EGFP fragment amplified from the plasmid pEGFP were ligated in frame into EcoRI and PstI-cleaved pPZP221 such that the NOS terminator was positioned behind EGFP.

All the constructs were confirmed by sequencing and transformed into wild type or mutant Arabidopsis plants using Agrobacterium tumefaciens strain ABI and the floral dipping method [57]. T1 transgenic plants were selected either on 1/2 MSNS plates containing 50 μg/mL kanamycin for the constructs in pPZP211 and 100 μg/mL gentamycin for the constructs in pPZP221, or on soil by spraying Liberty (glufosinate-ammonium) for the construct in pPZP221B. Homozygous transgenic plants were obtained for each construct based on segregation analysis of resistance to antibiotics or herbicide in the progeny.

**GUS Staining in Plants**

The Cyc1At-GUS reporter consists of the Cyc1At promoter plus the region coding for the first 150 amino acids of Cyc1At, including the cyclin destruction box (CDB). The CDB is fused in frame to GUS, so that CDB-GUS protein is degraded at the end of mitosis [29]. To visualize GUS reporter activity, seedlings were incubated in 90% acetone for 1 hour at room temperature and washed three times with GUS staining buffer (100mM NaPO4, pH 7) for 5 min each. The wash buffer was then replaced with the GUS staining solution [29] containing 1 mg/ml of 5-bromo-4-chloro-3-indoyl-B-D-glucuronide X-Gluc (Research Organics

---

**Figure 5. TMK mutants display reduced sensitivity to applied auxins.** (A) Root length of light grown seedlings: isogenic wild type, tmk1; tmk4 and tmk1;tmk3;tmk4. (B) Hypocotyl length of etiolated seedlings: isogenic wild type, tmk1;tmk4 and tmk1;tmk3;tmk4. (C) Lateral root density of wild type and tmk1; tmk4; and tmk1; tmk3; tmk4 mutants in response to 5.0 μM IAA. (D) Reduced sensitivity of tmk1;tmk4 mutant to applied auxin as illustrated by expression of DR5:GUS after treatments with 0.1 μM IAA, 2,4-D, and NAA, for 24 hours. Control panel represents no treatment. doi:10.1371/journal.pone.0060990.g005
Cleveland, Ohio) and the samples were incubated 4–6 hours at room temperature in the dark. The reaction was stopped by rinsing with buffer and then replacing the staining buffer with 70% EtOH.

**Microscopic Analysis**

Plant materials for light microscopy were examined using a BX60 Olympus microscope with DIC optics (Olympus Optical Company, Tokyo, Japan). Photographic images were recorded using the Olympus DP70 system. For the TMK1::GFP analysis, we used a Bio-Rad MRC-1024 Laser Scanning Confocal Microscope with the 24-bit imaging software (Bio-Rad, Hercules, CA). Developing siliques and stamen filament epidermal cells were imaged with a Quanta 200 Scanning Electron Microscope (FEI Company, Hillsboro, Oregon). The size of epidermal pavement cells was determined from cyanoacrylate glue replicas of the adaxial leaf surface [58]. Measurements were taken from four fields of each leaf, which contained ten or more epidermal cells.

**Root Kinematic Analysis**

The Arabidopsis root kinematic analysis was performed as described previously [30]. Seedlings were grown under light conditions for various time intervals, and at specific times, whole mounts of roots were analyzed by microscopy. Cell lengths of all cells in a cortical cell file were plotted against distance from the tip. The root growth rate data and cell file analysis were combined to calculate cell production rate (cells/hour), cell residence time (hours) and average growth rate (1/h):

\[
\text{Cell production rate} = \text{root elongation rate (mm/hour)} \times \text{final cell size (mm/cell)}
\]

\[
\text{Cell residence time} = \text{Number of cells in elongation zone} / \text{cell production rate (hours)}
\]

Average growth rate = \[
\frac{\ln(\text{final cell size}) - \ln(\text{initial cell size})}{\text{cell residence time (\mu m/\mu m.h)} \times (\mu m - 1) - 1} \times 1/h
\]

**Lateral Root Analysis**

Seeds of \textit{tmk1};\textit{tmk4}, \textit{tmk1};\textit{tmk3};\textit{tmk4} and isogenic wild type were plated and cold treated for three days before placement in the lights. Three days after germination, seedlings were transferred to 1/2 MSNS plates containing different concentrations of hormones and grown vertically for another 4 days. The number of lateral roots was counted and lengths of roots were measured as described previously [39].

**Statistical Analysis**

Statistical tests were performed using Mstat 4.0 software (University of Wisconsin, Madison).

**Acknowledgments**

The authors dedicate this paper to the memory of mentor, colleague, and partner Anthony B. Bleecker. We thank Rick Amasino, Donna Fernandez, and Edgar Spalding for critical review of the manuscript, Amber Boynton for plant care, Claudia Lipke for help with photography, and Brad Binder for technical assistance in preparing the manuscript. Special thanks to Shihmin Shiu for Western blotting and phylogenetic analysis. We would also like to thank John Celenza for providing the Cyc1AGUS line, and Sebastian Bednarek and Chris Day the pCD614 and pZP221B vectors, respectively.

**Author Contributions**

Conceived and designed the experiments: ND WW SEP ABB. Performed the experiments: ND WW SEP. Analyzed the data: ND WW SEP ABB. Contributed reagents/materials/analysis tools: ND WW SEP ABB. Wrote the paper: ND WW SEP ABB.
25. Shiu SH (2001) Characterization of the receptor-like kinase TMK1 and the molecular evolution of the receptor-like kinase gene family in Arabidopsis thaliana. University of Wisconsin, Madison, PhD Thesis.

26. Van der Knaap E, Song WY, Ruan DL, Sauter M, Ronald PC, et al. (1999) Expression of a gibberellic-induced leucine-rich repeat receptor-like protein kinase in deepwater rice and its interaction with kinase-associated protein phosphatase. Plant Physiol 120: 559–570.

27. Cho HS, Pai HS (2000) Cloning and characterization of a TMK1-homologous receptor-like kinase in Tobacco. Mol Cells 10: 317–324.

28. Lewers KS, Nilmalagoda SD, Warner AL, Knap HT, Matthews BF (2001) Physical mapping of resistant and susceptible soybean genomes near the soybean cyst nematode resistance gene Rg1f. Genome 44: 1057–1064.

29. Donnelly PM, Bonetra D, Tsukaya H, Dengler RJ, Dengler NG (1999) Cell cycling and cell enlargement in developing leaves of Arabidopsis. Dev Biol 215: 407–419.

30. Bremmer GT, Baskin TJ (1998) Analysis of cell division and elongation underlying the developmental acceleration of root growth in Arabidopsis thaliana. Plant Physiol 116: 1515–1526.

31. Polymenis M, Schmidt EV (1999) Coordination of cell growth with cell division. Curr Opin Genet Dev 9: 76–80.

32. Gao FB, Raff M (1997) Cell size control and a cell-intrinsic maturation program in proliferating oligodendrocyte progenitor cells. J Cell Biol 138: 1367–1377.

33. Cockcroft CE, den Boer BGW, Healy JMS, Murray JAH (2000) Cyclin D and flower development by promoting cell proliferation. Development 127: 1491–1501.

34. Clark SE (2001) Cell signaling at the shoot meristem. Nat Rev Mol Cell Biol 2: 276–284.

35. Shpak ED, Berthiaume CT, Hill EJ, Torii KU (2004) Synergistic interaction of three ERECTA-family receptor-like kinases controls Arabidopsis organ growth and Bower development by promoting cell proliferation. Development 131: 1491–1501.

36. Hemyat K, Hofte H (2008) Novel receptor kinases involved in growth regulation. Curr Opin Plant Biol 11: 321–328.

37. Hematy KU, Mitsukawa N, Oosumi T, Matsuura Y, Yokoyama R, et al. (1996) Physical mapping of resistant and susceptible soybean genomes near the soybean cyst nematode resistance gene Rg1f. Genome 44: 1057–1064.

38. De Smet I, Vo U, Jurgens G, Beeckman T (2009) Receptor-like kinases shape iterative plant development. EMBO J 28: 3923–3936.

39. Jones AM, Im KH, Savka MA, Wu MJ, DeWitt NG, et al. (1998) Auxin-dependent cell expansion mediated by overexpressed auxin-binding protein. Science 282: 1114–1117.

40. Arpiro P, Wu Y, LoCascio JC, Feldmann KA (1998) An Arabidopsis brassinosteroid-dependent mutant is blocked in cell elongation. Plant Cell 10: 219–230.

41. Hua J, Meyerowitz EM (1998) Ethylene responses are negatively regulated by a receptor gene family in Arabidopsis thaliana. Cell 94: 261–271.

42. Miao Y, Fischer KI (2000) Plant organ size control. ANTEGUMENTA regulates growth and cell numbers during organogenesis. Proc Natl Acad Sci U S A 97: 942–947.

43. Guendreau E, Traas J, Desnos T, Grandjean O, Caboche M, et al. (1997) Cellular basis of hypocotyl growth in Arabidopsis thaliana. Plant Physiol 114: 295–305.

44. Lally D, Ingrume P, Tong HY, He ZH (2001) Antisense expression of a cell wall-associated protein kinase, WARK1, inhibits cell elongation and alters morphology. Plant Cell 13: 1517–1531.

45. Bleeker AB, Kende H (2000) Ethylene: a gaseous signal molecule in plants. Annu Rev Cell Dev Biol 16: 1–18.

46. Alonso JM, Stepanova AN, Leisse TJ, Kim CJ, Chen H, et al. (2003) Genome-wide insertional mutagenesis of Arabidopsis thaliana. Science 301: 653–657.

47. Shiu SH (2001) Characterization of the receptor-like kinase TMK1 and the ethylene receptor family in Arabidopsis thaliana. Plant Physiol 124: 651–659.

48. Pfaffl M, Schwarz F, Sauerwein H (1998) Quantiﬁcation of insulin-like growth factor-1 (IGF-1) mRNA: modulation of growth intensity by feeding results in inter- and intra-tissue-speciﬁc differences of IGF-1 mRNA expression in steers. Exp Clin Endocr Diab 106: 514–521.

49. Huynkiewicz P, Swah Z, Maliga P (1994) The small, versatile reverse genetics system. Plant Cell 6: 2519–2523.

50. Bleecker AB, Kende H (2000) Ethylene: a gaseous signal molecule in plants. Annu Rev Cell Dev Biol 16: 1–18.

51. Alonso JM, Stepanova AN, Leisse TJ, Kim CJ, Chen H, et al. (2003) Genome-wide insertional mutagenesis of Arabidopsis thaliana. Science 301: 653–657.

52. O’Malley RC, Rodriguez FI, Esch JF, Binder BM, O’Donnell P, et al. (2005) Ethylene-binding activity, gene expression levels, and receptor system output for ethylene receptor family members from Arabidopsis and tomato. Plant J 41: 651–659.

53. Woycik WR, Hall AE, O’Malley R, Bleecker AB (2003) Canonical histidine kinase activity of the transmitter domain of the ETR1 ethylene receptor from Arabidopsis is not required for signal transmission. Proc Natl Acad Sci U S A 100: 352–357.

54. Pfaffl M, Schwarz F, Sauerwein H (1998) Quantification of insulin-like growth factor-1 (IGF-1) mRNA: modulation of growth intensity by feeding results in inter- and intra-tissue-specific differences of IGF-1 mRNA expression in steers. Exp Clin Endocr Diab 106: 514–521.

55. Hajdukiewicz P, Svab Z, Maliga P (1994) The small, versatile reverse genetics system. Plant Cell 6: 2519–2523.

56. Bleecker AB, Kende H (2000) Ethylene: a gaseous signal molecule in plants. Annu Rev Cell Dev Biol 16: 1–18.

57. Alonso JM, Stepanova AN, Leisse TJ, Kim CJ, Chen H, et al. (2003) Genome-wide insertional mutagenesis of Arabidopsis thaliana. Science 301: 653–657.

58. O’Malley RC, Rodriguez FI, Esch JF, Binder BM, O’Donnell P, et al. (2005) Ethylene-binding activity, gene expression levels, and receptor system output for ethylene receptor family members from Arabidopsis and tomato. Plant J 41: 651–659.

59. Woycik WR, Hall AE, O’Malley R, Bleecker AB (2003) Canonical histidine kinase activity of the transmitter domain of the ETR1 ethylene receptor from Arabidopsis is not required for signal transmission. Proc Natl Acad Sci U S A 100: 352–357.

60. Pfaffl M, Schwarz F, Sauerwein H (1998) Quantification of insulin-like growth factor-1 (IGF-1) mRNA: modulation of growth intensity by feeding results in inter- and intra-tissue-specific differences of IGF-1 mRNA expression in steers. Exp Clin Endocr Diab 106: 514–521.

61. Huynkiewicz P, Swah Z, Maliga P (1994) The small, versatile reverse genetics system. Plant Cell 6: 2519–2523.