Arabidopsis *thickvein* Mutation Affects Vein Thickness and Organ Vascularization, and Resides in a Provascular Cell-Specific Spermine Synthase Involved in Vein Definition and in Polar Auxin Transport[1]

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Polar auxin transport has been implicated in the induction of vascular tissue and in the definition of vein positions. Leaves treated with chemical inhibitors of polar auxin transport exhibited vascular phenotypes that include increased vein thickness and vascularization. We describe a recessive mutant, *thickvein* (*tkv*), which develops thicker veins in leaves and in inflorescence stems. The increased vein thickness is attributable to an increased number of vascular cells. Mutant plants have smaller leaves and shorter inflorescence stems, and this reduction in organ size and height is accompanied by an increase in organ vascularization, which appears to be attributable to an increase in the recruitment of cells into veins. Furthermore, although floral development is normal, auxin transport in the inflorescence stem is significantly reduced in the mutant, suggesting that the defect in auxin transport is responsible for the vascular phenotypes. In the primary root, the veins appear morphologically normal, but root growth in the *tkv* mutant is hypersensitive to exogenous cytokinin. The *tkv* mutation was found to reside in the *ACL5* gene, which encodes a spermine synthase and whose expression is specific to provascular cells. We propose that *ACL5/TKV* is involved in vein definition (defining the boundaries between veins and nonvein regions) and in polar auxin transport, and that polyamines are involved in this process.

The formation of the venation pattern in leaves is ideal for examining signaling pathways that recognize and respond to spatial and temporal information, since the pattern is two-dimensional and heritable, and arises during the intercalary growth of the leaf primordium (for review, see Nelson and Dengler, 1997). Although the molecular mechanisms underlying the patterning of leaf venation are not known, the role of the phytohormone auxin in vascular differentiation (e.g. Esau, 1965; Sachs, 1981) and, more specifically, the role of polar auxin transport in procambial (vascular precursor) strand formation and differentiation have been well documented (e.g. Lomax et al., 1995; Gälweiler et al., 1998; Mattsson et al., 1999; Sieburth, 1999). Auxin is thought to be transported in a primarily basipetal manner throughout the plant body from primary sources of auxin synthesis such as young developing leaves (e.g. Lomax et al., 1995), although recent studies have shown that the shoot apical meristem and very young leaf primordia are auxin sinks to which auxin is acropetally transported (Reinhardt et al., 2000; Avsian-Kretchmer et al., 2002). The basipetal transport of auxin from the leaf margin is thought to direct the venation pattern in the leaf (Mattsson et al., 1999; Sieburth, 1999; Aloni, 2001; Avsian-Kretchmer et al., 2002). When this transport is inhibited either chemically (Goto et al., 1991; Okada et al., 1991; Gälweiler et al., 1998; Mattsson et al., 1999; Sieburth, 1999) or genetically by knocking out the *PIN FORMED-1* (*PIN1*) gene, which encodes a component of the auxin efflux carrier (Gälweiler et al., 1998; Müller et al., 1998; Mattsson et al., 1999; Steinmann et al., 1999), the leaves develop increased proliferation of veins near the leaf margin, suggesting that the leaf margin is a major source of auxin within the developing leaf.

A number of leaf venation pattern mutants have been characterized in Arabidopsis (*Arabidopsis thaliana*), many of which exhibit disruptions in the continuity of the vascular pattern, producing isolated, short stretches of veins. A subset of those also have been shown to have reduced polar auxin transport in the inflorescence stem (e.g. *lop1*, *mp*, *sfc*, and *axr6*; Carland and McHale, 1996; Przemeck et al., 1996; Deyholos et al., 2000; Hobbie et al., 2000). Conversely, many auxin response mutants have reported vascular defects in the leaf. For example, the *bdl* mutant has a phenotype similar but weaker than *mp* and *axr6* mutants (Hamann et al., 1999).

Not surprisingly, many of the leaf venation pattern mutants with auxin defects contain mutations in genes whose products are involved in auxin response or polar auxin transport. For example, the *MP* gene encodes a transcriptional regulator of the auxin response factor family (ARF5; Hardtke and Berleth, 1998). The *BDL* gene encodes a member of the

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Aux/indole-3-acetic acid (IAA) family of transcription factors that has been shown to bind to MP and inhibit its activity in yeast (IAA12; Hamann et al., 2002). The *AXR6* gene encodes a cullin/CDC53-like subunit of the SCF^{TIR1} ubiquiting ligase complex, which has been implicated in auxin-dependent degradation of Aux/IAA proteins (Gray et al., 2001; Hobbie et al., 2002). The *VAN7/GN/EMB30* gene encodes a guanine nucleotide exchange factor protein involved in the polarized localization of PIN1 (Steinmann et al., 1999; Koizumi et al., 2000).

Still, the characterization of other non-auxin-related leaf venation pattern mutants has implicated other hormones/signaling pathways in vascular patterning. For example, the *CVP1* gene encodes STEROL METHYLTRANSFERASE-2, an enzyme in the sterol biosynthetic pathway, suggesting that sterols may play a specific role in vascular patterning or a general role in membrane organization that is essential for the axialization of procambial cells (Carland et al., 2002). A related gene, *ORC*, encodes STEROL METHYLTRANSFERASE-1, which is required for the correct subcellular localization of the putative auxin efflux carriers PIN1 and PIN3 (Willemsen et al., 2003). The *VEP1/AWI31* gene encodes a polypeptide similar to animal proteins that contain death domains and are involved in apoptosis (Jun et al., 2002). The *PLS* gene encodes a short polypeptide with no significant homology with known proteins and is required for correct auxin-cytokinin homeostasis (Casson et al., 2002).

Here, we identified a recessive Arabidopsis mutant, *thickvein* (*tkv*), which develops thicker veins in leaves and in inflorescence stems. The increased vein thickness is attributable to an increased number of both xylem and phloem cells. Mutant plants also have smaller adult leaves and shorter inflorescence stems. The reduction in organ size and height is accompanied by an increase in organ vascularization, which appears to be attributable to an increase in the recruitment of cells into veins. Moreover, the *tkv* mutant exhibits reduced auxin transport in the inflorescence stem, reinforcing the notion that auxin transport is responsible for vein definition. In the primary root, the veins appear morphologically normal, but root growth in the *tkv* mutant is hypersensitive to exogenous cytokinin. The *tkv* mutation was found to reside in the *ACL5* gene, which encodes a putative spermine synthase and is expressed during vascular development in provascular cells (uncommitted meristematic cells with the potential to become vascular cells) and in procambial cells (meristematic precursors to vein cells).

RESULTS

tkv Mutant Exhibits Increased Vein Thickness and Organ Vascularization

The *tkv* mutant was first identified from a mutant screen by its increased thickness of major veins in juvenile rosette leaves (Fig. 1, A and B). This phenotype was more conspicuous in adult rosette leaves, which were also smaller than those of wild type and had more veins per square area (Fig. 1, E and F). Transverse sections of *tkv* juvenile leaves confirmed that both the midvein (Fig. 1J) and secondary veins (Fig. 1L) were thicker than those of the wild type (Fig. 1, I and K, respectively), and indicated that the increased vein thickness was attributable to an increased number of both xylem and phloem cells as well as an increased number of procambial cells in between (Fig. 1, N and O). Cell size for all the leaf cell types appears normal at least cross-sectionally (Fig. 1, J and L; data not shown). Since in normal leaf vascular development the number of procambial cells increases by longitudinal divisions in the procambial cells themselves and by addition of cells from surrounding regions (Esau, 1965), it is very likely that the increase in vascular cell numbers found in the *tkv* leaf veins is due to an increase in outside cell recruitment and/or cell divisions within the procambium.

On a gross level, the *tkv* mutant resembled wild type up to the adult phase of vegetative development (Fig. 2A), where it exhibited a size reduction in its adult leaves (Fig. 2B). Since normal adult vegetative development involves an increase in the extent of leaf venation, which is reflected in overall leaf size, the size reduction and dense venation found in *tkv* adult leaves further substantiates the idea that cell recruitment into veins is increased. Inflorescence stems in the *tkv* mutant were similarly affected. There was severe defect in the length of stem internodes and a reduction in the number of stem nodes (Fig. 2, B and C). Furthermore, transverse sections of the most apical and basal parts of the inflorescence stem indicated a slight increase in xylem and phloem cell numbers and a large increase in the cambial-like cells between the phloem and xylem (Fig. 3E). This increase in vascular cell number is analogous to what was found in leaves, and appears to be partially at the expense of pith cell numbers. Primary root growth in the mutant was normal (data not shown), and transverse sections through the root elongation zone indicated that vascular cell numbers were also normal (Fig. 3H).

tkv Has Reduced Auxin Transport in the Inflorescence Stem

The increased vein thickness and vascularization in *tkv* leaves are reminiscent of the vascular phenotypes found in leaves treated with chemical inhibitors of polar auxin transport (Mattsson et al., 1999; Sieburth, 1999). To test if the cellular basis of the *tkv* vascular phenotype is due to a reduced transport of auxin, we measured the basipetal transport of ^{14}C-IAA in excised inflorescence stems of both wild-type and *tkv* mutant plants. We found polar auxin transport in *tkv* stems to be 66.2% of that in wild type (Fig. 4B). Furthermore, the distribution of transported ^{14}C-IAA in the *tkv* stem segment resembled that of wild type

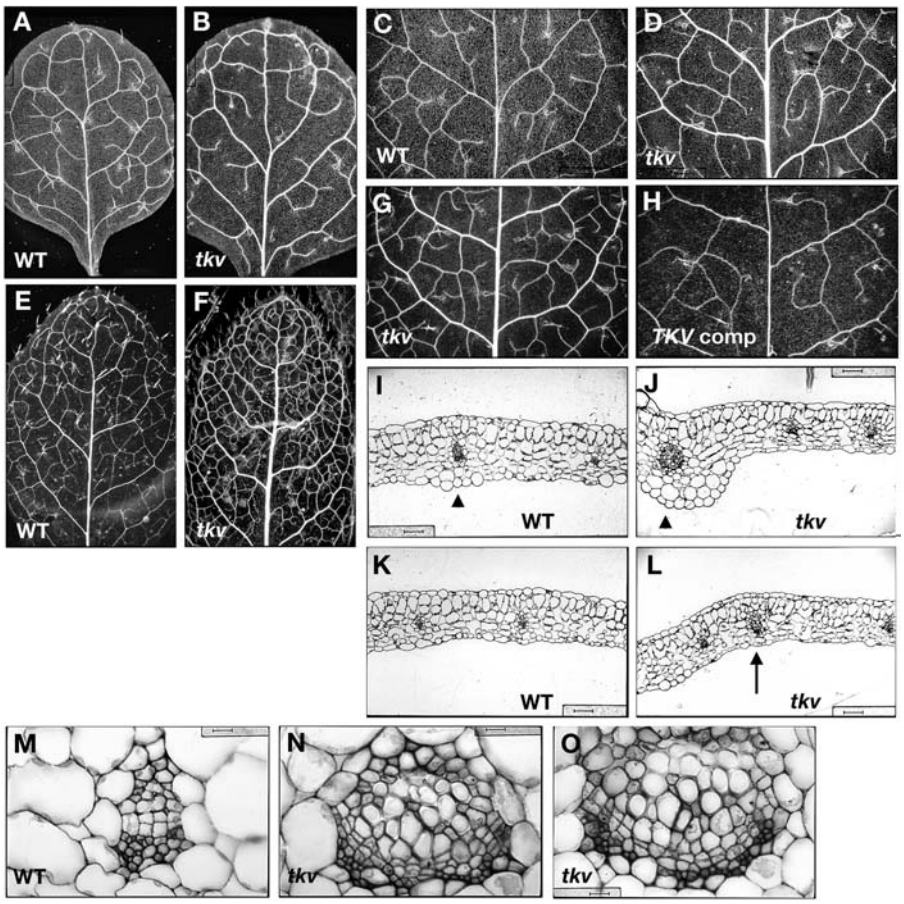

Figure 1. Increased vein thickness and vascularization in *tkv* leaves. A to H, Cleared leaves of wild type (A, C, and E), *tkv* (B, D, F, and G), and fully restored *tkv* containing wild-type copy of the *ACL5* transgene (H) were viewed under dark-field illumination to visualize the venation pattern. A and B, First pair of juvenile leaves. C, D, G and H, Second pair of juvenile leaves. E and F, Adult leaves. All leaves are in same magnification, and leaves used for comparison are developmentally equivalent (have similar number of secondary veins that form prominent arches that begin and end at the midvein) and representative of numerous samples. I to O, Transverse sections through the midvein (I, J, and M–O) and lateral veins (K and L) of fully expanded juvenile leaves. I, K, and M, Wild type. J, L, N, and O, *tkv*. Arrowheads indicate the midvein, and arrow indicates a secondary vein. In wild type, aside from the midvein, higher-order veins are indistinguishable by vein thickness. Scale bars in I to L = 50 μm, and scale bars in M to O = 16 μm.

(Fig. 4A), indicating that shortened internodes did not interfere with auxin transport. Unlike wild-type plants treated with polar auxin transport inhibitors (Okada et al., 1991) and other characterized auxin transport mutants exhibiting female sterility and/or floral bud deficiencies, e.g. *lop1* (22.7% of wild-type auxin transport; Carland and McHale, 1996), weak alleles of *mp* (31.6%; Przemeck et al., 1996), *pin1* (50%; Okada et al., 1991; Bennett et al., 1995), and *pid* mutant (74%; Bennett et al., 1995), *tkv* plants are fully fertile and produce normal flowers. The production of fertile, fully formed flowers suggests that IAA content and auxin response in the *tkv* inflorescence apex are sufficient for organ formation, since exogenous application of IAA on *pin1* and *pid* inflorescence apices can induce organ formation (Reinhardt et al., 2000, 2003).

This auxin-related result encouraged us to test the *tkv* mutant for a number of hormonal responses by measuring primary root growth in media containing exogenous amounts of auxin (IAA and 2,4-dichlorophenoxyacetic acid), cytokinin (6-benzylaminopurine), epi-brassinolide, gibberellin, or abscisic acid. Only exogenous cytokinin produced an altered hormone response in the *tkv* mutant, severely reducing root growth relative to that of wild type (Fig. 5). This cytokinin hypersensitivity is intriguing because of the normal vascular morphology of the *tkv* root.

tkv Mutation Resides in *ACL5* Gene, Which Encodes a Spermine Synthase

A map-based cloning strategy was used to isolate the gene. Genetic analysis of F_2 progeny indicated that the *tkv* mutant phenotype segregated as a single recessive locus. The *tkv* mutation was mapped initially between simple sequence length polymorphic markers nga106 and nga139 on the top of chromosome 5, and then finely mapped to a region spanned by bacterial artificial chromosomes F7K24 and T20D1 that contained nine predicted genes. One of the genes, *ACAULIS5* (*ACL5*), encodes a spermine synthase involved in polyamine biosynthesis, and null mutations in the gene resulted in a severe defect in the elongation of the stem internode (Hanzawa et al., 2000). Since the *tkv* mutant has a similar internodal elongation defect, the *ACL5* gene in the mutant was selected for sequencing, and was found to contain a base pair deletion at the start of exon 7 (Fig. 6A). The resulting frameshift in the mRNA would encode a premature stop codon after 19 amino acids (Fig. 6B). The *ACL5* gene consists of nine exons that span a 1.9-kb genomic region (Fig. 6A). Introduction of the *ACL5* promoter-driven wild-type *ACL5* transgene into the *tkv* mutant fully complemented the mutant phenotype with respect to both the thick veins and the short internodes for five

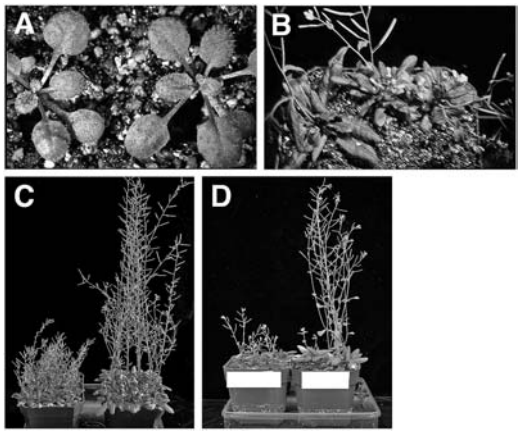

Figure 2. Shortened inflorescence stems of *tkv* mutant. A, Two-week-old wild-type (left) and *tkv* (right) plants. B, Top view of 4-week-old wild-type (left) and *tkv* (two on right) plants. C, Fully grown *tkv* (left) and wild-type (right) plants. D, Fully grown *tkv* plants (left) and *tkv* plants containing the wild-type *ACL5* transgene (right).

independent transgenic lines (Figs. 1, C and F, and 2D, respectively).

ACL5 Expression Is Provascular/Procambial-Specific

Hanzawa et al. (2000) had shown that *ACL5* expression is up-regulated in the *acl5-1* mutant (lesion shown in Fig. 6B), suggesting that *ACL5* expression may be under negative-feedback control. The expression of the *ACL5* gene was examined in wild-type and *tkv* plants by reverse transcription (RT)-PCR and was found to be similarly up-regulated in *tkv* mutant plants (Fig. 7).

The expression pattern of *ACL5* was visualized by RNA in situ hybridizations as well as histochemical staining of plants containing a transgenic copy of the *ACL5* promoter driving the β-glucuronidase (GUS) reporter gene. *ACL5* expression was found throughout early globular-staged embryos and persisted throughout embryogenesis until bent cotyledon-staged embryos where the expression domain was delimited to procambial cells (Figs. 8A and 9, B–D). The procambial-specific expression pattern continues during primary root development (Figs. 8, E and G, 9I) and early leaf development after an initially broad expression domain in leaf primordia (Figs. 8C and 9, E–H). This expression pattern holds true during inflorescence development. Transverse sections through GUS-stained inflorescence stems revealed GUS expression throughout the vascular bundle of the stem as well as the procambial strands of axillary buds (Fig. 9, K–M). Recently, an independent group performed digital in situ hybridization and conventional RNA in situ hybridization of *ACL5* on primary roots and found its expression to be specific to and abundant in procambial cells (Birnbaum et al., 2003).

DISCUSSION

TKV Is Involved in Vein Definition

In plants, vein formation in the leaf is closely linked to cell proliferation in the leaf since much of the minor venation is formed between older veins during the intercalary, extension growth of the leaf blade (Esau, 1965). Many Arabidopsis leaf venation mutants with reduced venation have also reduced leaf size. They include mutants *lop1* (Carland and McHale, 1996), *cvp1* (Carland et al., 1999), *scf* (Deyholos et al., 2000), *van7* (Koizumi et al., 2000), *mp* (Przemeck et al., 1996), *axr6* (Hobbie et al., 2000), and *vep1* (Jun et al., 2002). By contrast, the *tkv* mutant has more venation per leaf surface area in adult leaves and more vascular cell files per vein despite the smaller adult leaves and shorter internodes. The first four rosette leaves are considered juvenile leaves and were indistinguishable in size

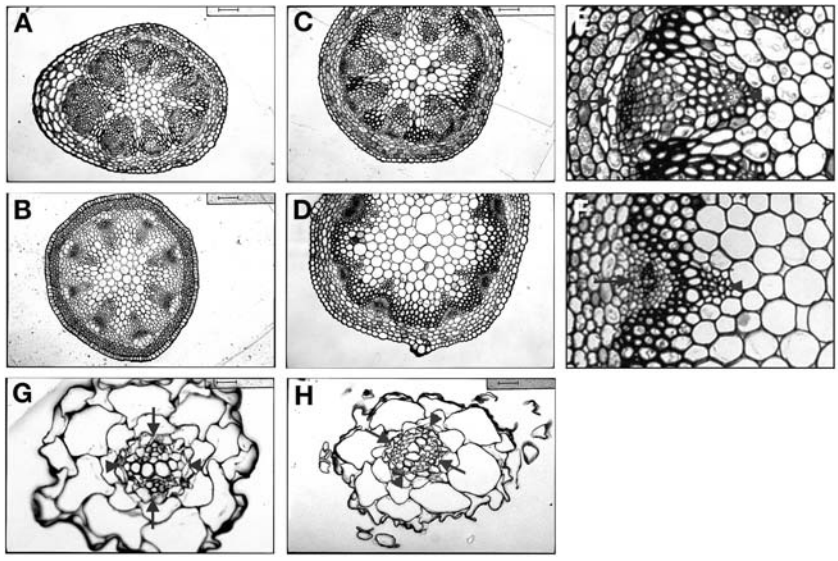

Figure 3. Increased cambium in inflorescence stems of *tkv* mutant. A to F, Transverse sections through the apical end of the inflorescence stem (just below the youngest silique; A and B) and the basal end of the inflorescence stem (C, D, E, and F). A, C, E, *tkv*. B, D, F, Wild type. E and F are enlargements of a vascular bundle in C and D, respectively. Opaque cells inside of the phloem are cambial-like cells and possibly include xylem parenchyma cells. Arrowheads point to xylem, and arrows point to phloem. Scale bars in A, B, C, D, G, and H = 50 μm, and scale bars in E and F = 25 μm. G and H, Transverse sections through the primary root of week-old seedlings in the root elongation zone, where root hairs emerge. G, Wild type. H, *tkv*. Arrowheads point to xylem axes, and arrows point to phloem cells. Scale bars = 16 μm.

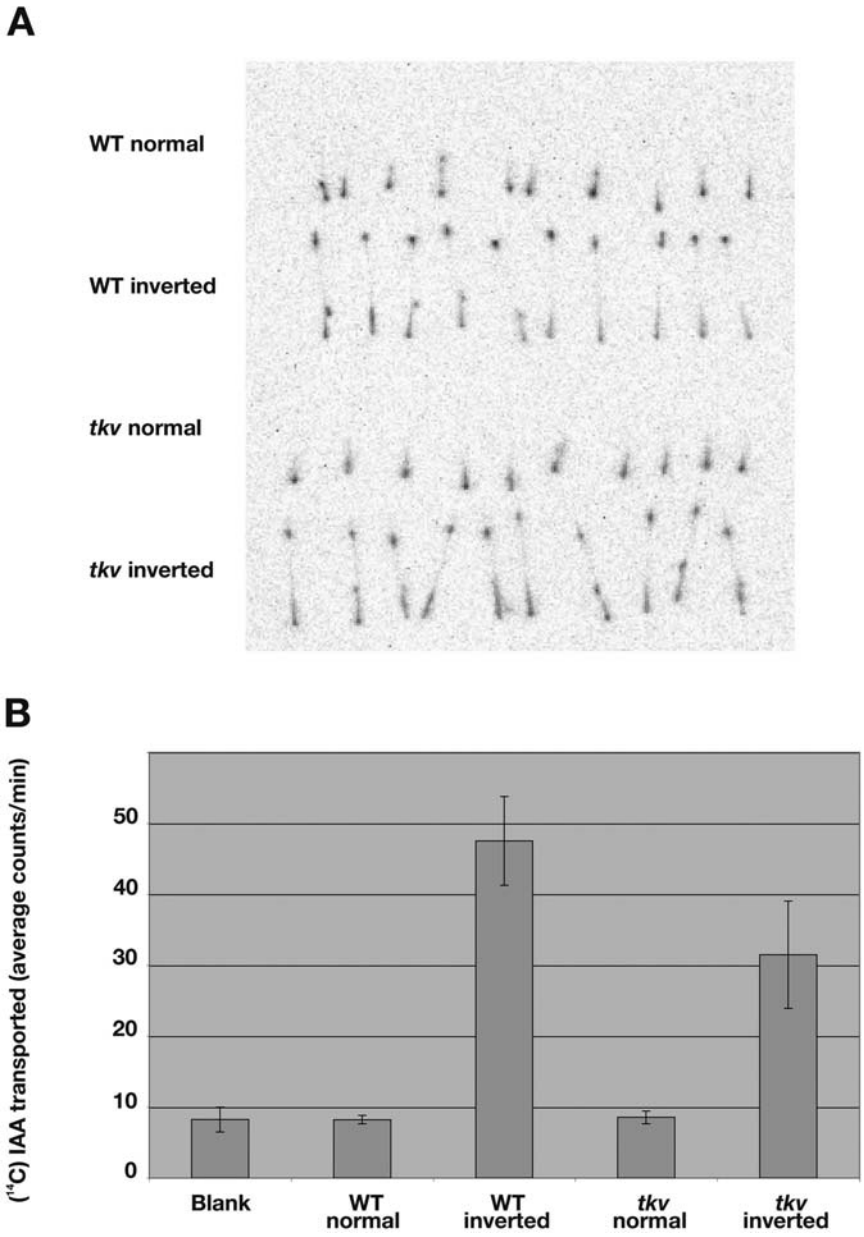

Figure 4. Reduced polar auxin transport in inflorescence stems of *tkv* mutant. A, The basipetal (inverted orientation) and acropetal (normal orientation) transport of ^{14}C-IAA in excised inflorescence stem segments were visualized using the Fujix image analyzer. B, Average cpm of ^{14}C-IAA transported to the distal end of the stem segments were calculated from 80 samples of each genotype from four separate experiments. Error bars indicate SD.

between *tkv* mutant and wild type. Subsequent rosette leaves are considered adult leaves and arise during the switching of the shoot meristem from vegetative phase to reproductive phase. Although these leaves were smaller sized in the *tkv* mutant, leaf cell size appeared normal at least cross-sectionally. Similar observations were made with the *acl5* mutant, whose reduced adult leaf size was attributed mainly to reduced cell elongation in the petioles after flowering (Hanzawa et al., 1997). The increased venation in the *tkv* mutant appears to be at the expense of a cell population normally recruited to mesophyll cell development. Alternatively, it may also arise from increased cell divisions within the procambium. If that is the case, these increased divisions have a suppressive effect on the proliferation of surrounding cells, for instance, by siphoning off resources (hormones, nutrients, etc.) necessary for proliferation. Whether the *tkv* mutation solely affects cell recruitment into veins, or the balance between vein formation and cell proliferation in the surrounding cells, or both, it does appear that *TKV* is involved in a mechanism that defines the boundaries between veins and nonvein regions. This mechanism may be analogous to the lateral inhibition mechanism involving Notch signaling to establish the correct vein thickness in Drosophila wings (de Celis et al., 1997). We propose that vein definition is achieved through the polar auxin transport with its built-in lateral inhibitory effect on auxin transport capacities of surrounding cells and inductive effect on

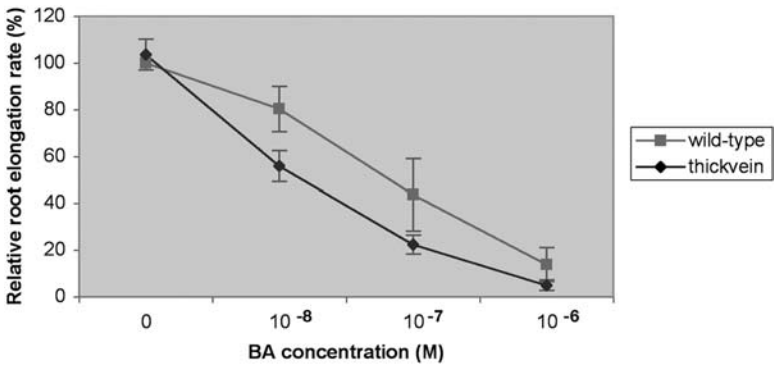

Figure 5. Root growth in *tkv* mutant is hypersensitive to exogenous cytokinin. Relative root elongation rates of wild-type (squares) and *tkv* mutant (diamonds) 10-d-old seedlings. Mean values for 100% root elongation were determined on Murashige and Skoog medium containing no 6-benzylaminopurine (BA). Error bars indicate SD.

procambium formation and differentiation on transporting cells (see ''canalization of signal flow hypothesis''; Sachs, 1981), and that the *tkv* mutant documents this link.

Finally, because auxin transport is hypothesized to be involved in the induction of procambial cells with their characteristic narrow elongate shape, there may be a causal connection between rate of auxin transport and extent of cell elongation along the longitudinal axis. If that is the case, then the reduced inflorescence stems in the tkv mutant can be attributed to a reduced rate of auxin transport through procambial cells and xylem parenchyma cells.

TKV Is Involved in Polar Auxin Transport

Like the *tkv* mutant, four other Arabidopsis mutants have been reported to display reduced auxin transport in stem segments (in order of increasing severity: *pid*, *pin1*, *mp*, and *lop1*; Okada et al., 1991; Bennett et al., 1995; Carland and McHale, 1996; Przemeck et al., 1996). However, unlike these mutants, the *tkv* mutant

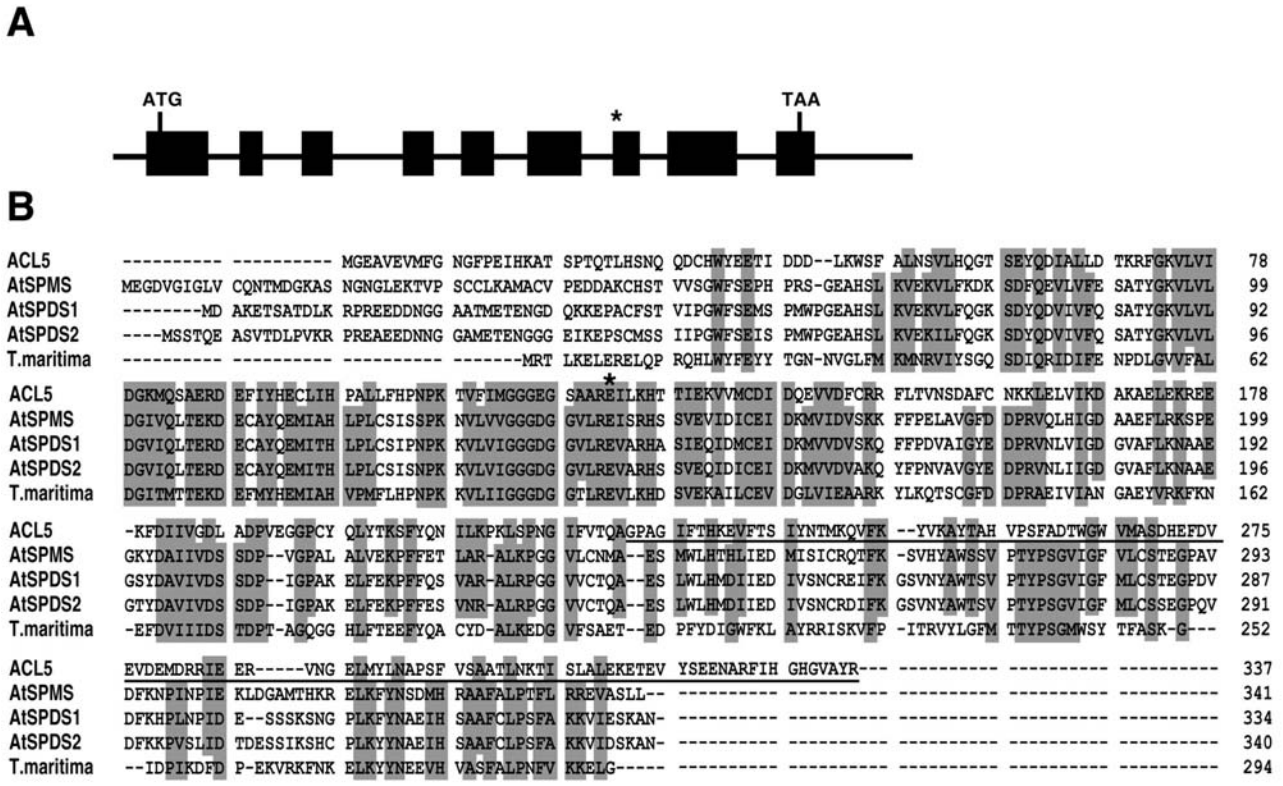

Figure 6. *ACL5* encodes a spermine synthase involved in polyamine biosynthesis. A, Genomic structure of the *ACL5* gene. Exons are indicated by black boxes. Asterisk indicates the missing nucleotide in *tkv* allele. B, Alignment of the deduced amino acid sequences of ACL5, AtSPDS1, AtSPDS2, AtSPMS, and *Thermotoga maritima* spermidine synthase (for which a crystal structure was solved [Korolev et al., 2002]). Amino acids affected by the *tkv* mutation are underlined, and the Glu residue mutated in *acl5-1* allele is marked with an asterisk.

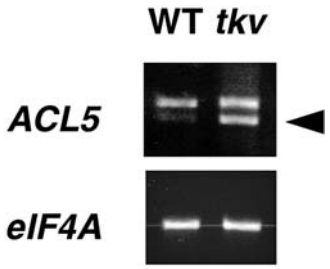

Figure 7. *ACL5* mRNA expression analyses. Total RNA from wild-type and *tkv* inflorescences were reverse transcribed and PCR amplified using primers to the 3′ end of *ACL5* cDNA. RT-PCR products were run on an ethidium bromide-stained gel. PCR-amplified *eIF4A* was the loading control. Arrowhead marks spliced transcript.

produced fully fertile and well-formed flowers despite the fact that *tkv* mutant stems displayed auxin transport capacities between those of *pid* and *pin1* mutants. This indicates that IAA content and auxin response in the *tkv* inflorescence apex are sufficient for organ formation, since exogenous application of IAA on *pin1* and *pid* inflorescence apices can induce organ formation (Reinhardt et al., 2000, 2003).

Similarly in the *tkv* root, auxin response and vascular formation appeared normal, which would suggest that *TKV* gene activity is dispensable in roots except for the fact that the roots exhibited cytokinin hypersensitivity. In addition to the well-documented role of polar auxin transport in vascular induction and procambium differentiation, cytokinin also has been implicated in vascular development, functioning in a complex and sometimes synergistic relationship with auxin (for review, see Lyndon, 1990; Klee and Lanahan, 1995). *TKV*'s provascular gene expression pattern localizes this cytokinin hypersensitivity to the vasculature of the root. Because of the nature of the *TKV* gene product, this defect is likely to be secondary. Furthermore, the otherwise normal morphology suggests the existence in the root of additional gene functions overlapping with that of *TKV*.

Unlike *lop1* and *mp* mutants, which have reduced vascular systems (Carland and McHale, 1996; Przemeck et al., 1996), the *tkv* mutant has increased formation of xylem, phloem, and procambium, as well as increased vascularization in leaves, all of which correspond to vascular features induced by auxin transport inhibitors such as 1-*N*-naphthylphtalamic acid (NPA; Mattsson et al., 1999; Sieburth, 1999). The reduced auxin transport found in *mp* and *lop1* mutant stem segments is likely to result from vascular discontinuity and/or reduced number of developing vascular strands, rather than from impaired auxin transport capacities of individual cells. In contrast to *lop1*, *mp*, *pin1*, and *tkv* mutants, the *pid* mutant (which exhibited the least reduction in auxin transport and only in stems that had ceased elongating) exhibited very mild vascular defects that appeared only in flowers, suggesting that the regulation of vascular development and/or auxin transport is not the primary task of *PID* (Christensen et al., 2000; Benjamins et al., 2001).

Despite the superficial similarity between *pid* mutant inflorescences to those of *pin1*, the vascular defects found in *pin1* mutant leaves are similar to but weaker than those found in the leaves of NPA-treated plants (Mattsson et al., 1999). Furthermore, the *PIN1* gene encodes a putative membrane protein that is located at the basal end of auxin transport-competent cells, indicating that the *PIN1* gene product is part of the general auxin efflux mechanism that is inhibited by the auxin transport inhibitor NPA (Lomax et al., 1995; Gälweiler et al., 1998). However, the vascular features

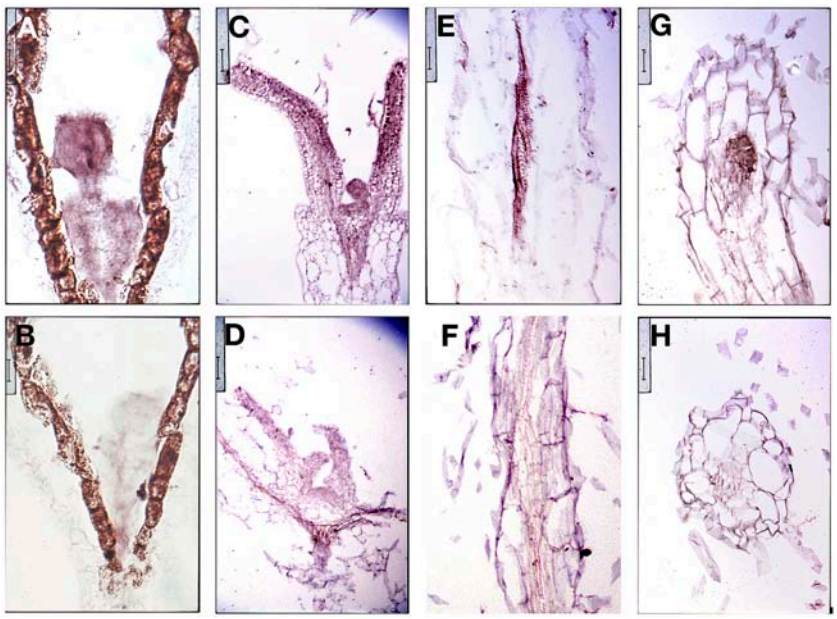

Figure 8. *ACL5* expression is associated with provascular/procambial cells. A to H, Sections were hybridized with sense (B, D, F, H) and antisense (A, C, E, G) DIG-labeled *ACL5* RNA probe. A and B, Longitudinal sections through globular-staged embryos. Scale bars = 12.5 μm. C and D, Longitudinal sections through leaf primordia of week-old seedlings. Scale bars = 50 μm. E and F, Longitudinal sections through root of week-old seedlings. Scale bars = 25 μm. Note the DIG-labeled vasculature. G and H, Transverse sections through root of week-old seedlings. Scale bars = 25 μm.

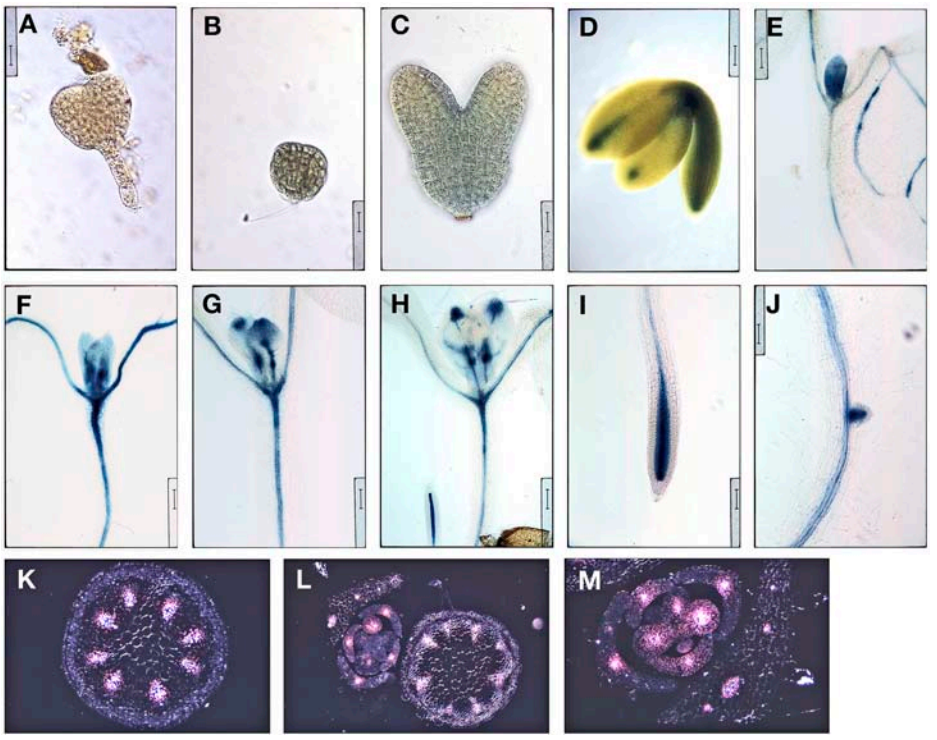

Figure 9. *ACL5* promoter activity is associated with provascular/procambial cells. A, Nontransgenic globular-staged embryo was stained for GUS activity. Note the absence of GUS stain. Scale bar = 16 μm. B to M, Wild-type plants containing a transgenic copy of the *ACL5* promoter-driven *GUS* reporter gene were stained for GUS activity and viewed under bright-field illumination unless noted otherwise. B, Globular-staged embryo. Scale bar = 16 μm. C, Torpedo-staged embryo. Staining is weak and diffuse. Scale bar = 16 μm. D, Bent cotyledon-staged embryo. Scale bar = 62.5 μm. E to J, Cleared GUS-stained week-old seedlings were arranged in developmental order. Note that GUS stain gradually localizes to and around PC cells. Scale bars = 100 μm. K to M, Transverse sections through inflorescence stems and axillary buds were viewed under dark-field illumination to visualize GUS staining, which appears pink. Note that GUS staining is localized to developing vasculature. Scale bars = 50, 100, and 50 μm, respectively.

found in *tkv* mutant leaves correspond more so in detail and in severity to those found in NPA-treated plants. Furthermore, this correspondence extends to inflorescence stems, whereas *pin1* mutant stems have only increased xylem formation (Gälweiler et al., 1998). Because *PIN1* is a member of a large gene family, it is likely that there are related gene products with partially overlapping functions that can complement the loss of *pin1* function in the root and in the stem and thereby account for the imperfect correspondence between *tkv* and *pin1* mutant stems and between NPA-treated leaves and *pin1* mutant leaves, respectively. All of this suggests that *TKV*'s role in polar auxin transport may be in the general efflux mechanism that is sensitive to NPA inhibition.

Role of Polyamines in Polar Auxin Transport

Numerous studies have implicated polar auxin transport in the induction of vascular tissue and in the definition of vein positions. The increased thickness of veins and vascularization of leaves found in the *tkv* mutant can be phenocopied in wild type by the chemical treatment of auxin transport inhibitors (Mattsson et al., 1999; Sieburth, 1999). Similarly, the mutant also exhibits a reduction of auxin transport through its short inflorescence stem (with its thick vascular bundles). The presence of fertile and normal-looking flowers in the mutant suggests that auxin response is not generally affected in the shoot apex and that the auxin transport defect is specific and may be primarily responsible for the vascular phenotypes. The nature of the *TKV* gene product suggests that the relationship between polar auxin transport and vein definition may involve spermine. The *tkv* mutation resides in the *ACL5* gene, which was previously found to encode a putative spermine synthase. Although the in vivo function of ACL5 is still unclear, *ACL5* expression is auxin inducible (Hanzawa et al., 2000) and provascular/procambial specific (this work), further implicating ACL5 enzyme activity in the polar transport of auxin through veins.

Auxin is not the only growth factor that is actively transported. Polyamines, which are small, evolutionarily ancient polycationic amines that are ubiquitously present in bacteria, animals, and plants, are necessary for normal cell growth and development (Cohen, 1998) and have been implicated in many cellular processes such as cell proliferation, transcription, translation, and chromatin structure (Marton and Morris, 1987; Cohen, 1998; Pollard et al., 1999). In animal systems, polyamines have been shown to move between cells via specific transporters in an energy-dependent manner (Seiler and Dezeure, 1990). In Arabidopsis, biochemical and physiological data suggest that polyamines can be transported through xylem and phloem (Caffaro et al., 1993; Antognoni et al., 1998). Because the knockout of ACL5 function had no effect on the endogenous levels of free and conjugated polyamines in Arabidopsis (Imai et al., 2004), it remains to be seen whether the active transport (and not the levels) of polyamines (more specifically, spermine) is involved in vein definition and/or auxin transport.

Spermine is one of three plant polyamines (spermidine and putrescine are the other two), and in plants, polyamines, chiefly spermidine, were found to

modulate the activity of KAT1-like inward-rectifying K^+ channels and thus to regulate the closure of guard cells during stress (Liu et al., 2000). Still, the specifics regarding the developmental function of polyamines remain a mystery. Most plants form putrescine directly from Orn and indirectly from Arg. Spermidine is formed from putrescine and spermine from spermidine by successive addition of aminopropyl groups by spermidine and spermine synthases, respectively. Arabidopsis contains two spermidine synthase genes (*AtSPDS1* and *AtSPDS2*) and two spermine synthase genes (*ACL5* and *AtSPMS*), and all are likely to encode cytoplasmic enzymes (Hanzawa et al., 2002). Of the four, *ACL5* is the most dissimilar (Hanzawa et al., 2002), suggesting its unique genetic origin. Recently, a polyamine metabolon (a complex consisting of successive enzymes) has been isolated in Arabidopsis, and it contained AtSPDS1, AtSPDS2, and AtSPMS, but not ACL5 (Panicot et al., 2002). All three enzymes found in the metabolon directly interacted with one another in yeast and in vitro, but no interaction was found in a direct two-hybrid test with ACL5 (Panicot et al., 2002), suggesting that ACL5 may have a divergent function or role in development. Although ACL5 has been shown to function as a spermine synthase in *Escherichia coli* (Hanzawa et al., 2000), the knockout of ACL5 function had no effect on the endogenous levels of free and conjugated polyamines in Arabidopsis (Imai et al., 2004), suggesting that ACL5 may have a very specific or altogether different in vivo function.

MATERIALS AND METHODS

Plant Materials and Growth Conditions

Arabidopsis (*Arabidopsis thaliana*) ectoypes Columbia (Col-O) and Landsberg carrying the *erecta* mutation (L*er*) were used for comparison with mutant plants and for crosses. Seeds were grown under constant white light (approximately 300 μE m^{-2} s^{-1}) either on 0.75% agar media consisting of Murashige and Skoog (1962) basal salts, Haughn and Somerville (1986) nutrient solution, 0.5 g/L MES, and 10 g/L Suc, or on soil (3:1 mix of Metro-Mix 200 to vermiculite; Scotts, Marysville, OH).

Mutant Isolation

A visual screen for venation pattern mutants was performed on diepoxybutane-mutagenized M_2 Arabidopsis (ecotype Col-O) seeds. Briefly, hydrated seeds were incubated in 18 mM diepoxybutane (Sigma, St. Louis) for 4 h and washed extensively before planting (M_1 generation). M_2 seeds, the progeny of self-fertilized M_1 plants, were pooled from every 10 M_1 lines and screened about 2 weeks after germination. One to two rosette leaves from each M_2 plant were fixed in 3:1 ethanol to acetic acid, dehydrated in an ethanol series, cleared in Hemo-De (Fisher-Scientific, Loughborough, Leicestershire, UK), mounted on slides with 2:1 Permount (Fisher-Scientific) to xylene, and viewed under dark-field optics for alterations in the venation pattern. Putative venation pattern mutants were confirmed by a secondary screen and backcrossed twice to wild-type Col-O plants for further phenotypic characterization.

Histochemical Localization of GUS Activity and Histological Analyses

Plant tissues were stained for GUS activity overnight at 37°C in GUS buffer, 20% methanol, and 0.5 mg/mL X-Gluc (5-bromo-4-chloro-3-indolyl-β-D-glucuronidase) as described by Malamy and Benfey (1997). For observation of whole mounts, both stained and unstained tissue were fixed, cleared, and mounted on slides as described above. Specimens were examined with a Zeiss Axiophot microscope (Oberkochen, Germany). Tissues used for RNA in situ hybridizations were fixed overnight at 4°C in formaldehyde-acetic acid (3.7% formaldehyde, 50% ethanol, and 5% acetic acid). For histological analysis, fixed dehydrated specimens were embedded in Spurr's media (EM Sciences, Ft. Washington, PA) or in Paraplast Plus (Fisher-Scientific). Plastic sections (2 μm) were cut on a Sorvall MT2-B ultramicrotome (Newtown, CT) with glass knives and were stained briefly with 1% toluidine blue. Paraffin sections (8 μm) were cut on a model 820 microtome (Arthur H. Thomas, Philadelphia).

Polar Auxin Transport Assay

Polar auxin transport in inflorescence axes was measured using a modification of the method described by Okada et al. (1991). Two-centimeter-long stem segments from inflorescence axes of wild-type and *tkv* mutant plants between 1 and 2 weeks after bolting were taken from near the rosette to the first flower-bearing node. Segments were placed in either normal or inverted orientation in 1.75-mL tubes containing 30 μL of half-strength Murashige and Skoog (1962) basal medium containing 1.45 μM ^{14}C-labeled IAA (Sigma), incubated in closed tubes at room temperature for 19 h, washed three times in IAA-free medium, arrayed between sheets of plastic wrapping, and exposed to a phosphorimaging plate for 12 h. Distribution of labeled IAA in the segments were visualized by a Fujix BAS2000 image analyzer (Fuji Photo Film, Tokyo). After exposure, 5-mm-long segments were cut from the upper end of the stem segments and incubated overnight in Opti-fluor scintillation fluid (Packard Bioscience, PerkinElmer, Shelton, CT). Radioactivity released by the upper stem segments were counted by a Beckman LS6000IC scintillation counter (Beckman Instruments, Munich).

Genetic Mapping and Plant Transformation Vector Construction

Plants homozygous for the *tkv* mutation were crossed to wild-type L*er* plants to generate an F_2 mapping population. DNA from 20 mutant plants were used with simple sequence length polymorphic markers (Bell and Ecker, 1993; Ponce et al., 1998) to map the chromosomal location of the *tkv* mutation, and DNA from approximately 500 mutant plants, respectively, were used with Cereon's insertion/deletion and single-nucleotide polymorphism markers (Jander et al., 2002) to map *tkv* to a region spanned by a single bacterial artificial chromosome.

To generate the complementation construct, 4.24 kb of *ACL5* genomic sequence, which includes 2.10 kb of upstream sequence (minus the stop codon), was PCR-amplified and subcloned into *Hin*dIII/*Stu*I sites of the PJIM19smGFP binary vector upstream of the *smGFP* gene. To generate the reporter construct, 2.13 kb of upstream sequence was PCR-amplified and subcloned into *Hin*dIII/*Bam*H1 sites of the PBI101 binary vector upstream of the GUS reporter gene. Both contructs were sequenced for errors and introduced into *tkv* mutant and wild-type plants, respectively, via Agrobacterium-mediated floral dip method (Clough and Bent, 1998), and transformants were selected on agar media containing 50 mg/mL kanamycin.

Total RNA Isolation and RT-PCR

Total RNA was isolated from inflorescences including open flowers and 2-cm-long stems near the apex with TRIzol (Gibco-BRL, Gaithersburg, MD) according to manufacturer's instructions. Two micrograms of total RNA was reverse transcribed with 200 units of SuperscriptII (Invitrogen, Carlsbad, CA). The resulting cDNA:RNA hybrids were treated with 10 units of DNase I (Roche, Indianapolis) for 30 min at 37°C, purified on Qiaquick PCR column (Qiagen USA, Valencia, CA), and used as template to PCR-amplify *ACL5* (40 cycles) and *eIF4A* (39 cycles). PCR conditions are as follows: 94°C for 15 s, 52°C for 15 s, and 72°C for 15 s. PCR products (350–400 bp) were electrophoresed in 1.5% agarose gel and visualized with a Gel Doc 2000 system (Bio-Rad, Hercules, CA).

RNA in Situ Hybridization

ACL5 cDNA (nucleotides 996–1,106 relative to ATG) was PCR amplified using primers containing engineered T3 and T7 RNA polymerase promoter

sites and was used as template to generate dioxigenin (DIG)-labeled sense and antisense RNA probes. In situ hybridizations and labeling reactions were carried out as described by Jackson (1991) with some modifications, which were described by Clay and Nelson (2002).

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