Intercellular trafficking of a KNOTTED1 green fluorescent protein fusion in the leaf and shoot meristem of Arabidopsis

Jae Yean Kim*, Zhuang Yuan*, Michelle Cilia†, Zainab Khalfan-Jagani*§, and David Jackson†¶

1Cold Spring Harbor Laboratory, 1Watson School of Biological Sciences, 1 Bungtown Road, Cold Spring Harbor, NY 11724

Edited by Robert Haselkorn, Integrated Genomics, Inc., Chicago, IL, and approved January 7, 2002 (received for review September 13, 2001)

Dominant mutations in the maize homebox gene knotted1 (kn1) act nonautonomously during maize leaf development, indicating that Kn1 is involved in the generation or transmission of a developmental signal that passes from the inner layers of the leaf to epidermal cells. We previously found that this nonautonomous activity is correlated with the presence of KN1 protein in leaf epidermal cells, where KN1 mRNA could not be detected. Furthermore, KN1 protein expressed in Escherichia coli and labeled with a fluorescent dye can traffic between leaf mesophyll cells in microinjection assays. Here we show that green fluorescent protein (GFP)-tagged KN1 is able to traffic between epidermal cells of Arabidopsis and onion. When expressed in vivo, the GFP–KN1 fusion trafficked from internal tissues of the leaf to the epidermis, providing the first direct evidence, to our knowledge, that KN1 can traffic across different tissue layers in the leaf. Control GFP fusions did not show this intercellular trafficking ability. GFP–KN1 also trafficked in the shoot apical meristem, suggesting that cell-to-cell trafficking of KN1 may be involved in its normal function in meristem initiation and maintenance.

T
he plasticity of plant development and evidence from mosaic analysis indicate that cell fate is generally determined late in plant development through positional information (1–3). Cell-to-cell communication provides the means by which cells determine their position in relation to short- and long-range signals. These signals could pass through the apoplas, as for example as secreted ligands, or the symplasm, through cytoplasmic channels called plasmodesmata (PDs) that connect the majority of plant cells (4–7).

Plasmodesmal channels are bounded by a sleeve of plasma membrane and traversed by a tube of appressed endoplasmic reticulum (ER). Passage of small molecules and regulated transport of macromolecules occur through the cytoplasmic channel between the ER and plasma membrane (4–7). PDs are classified as primary, if formed during cytokinesis, or secondary, when synthesized through an existing cell wall; this latter class is important for connecting cells that do not share a recent division wall, for example those in adjacent layers of the shoot apical meristem (SAM) (8). During later stages of leaf development, PDs can also be modified so that the simple channels become branched, and this modification is correlated with changes in PD size-exclusion limit (SEL) and ability to traffic specific proteins (9–11).

Many viruses encode movement proteins (MPs) that localize to PDs and traffic themselves and MP–viral nucleic acid complexes between cells (12, 13). It is believed that MPs ride on an endogenous intercellular trafficking pathway; this hypothesis is supported by the discovery of a plant MP-related protein, PP16, which is expressed in the phloem and can traffic itself and RNA through PDs (14). Evidence for endogenous mRNA trafficking in the phloem is also evident from SUCROSE TRANSPORTER 1 mRNA localization studies (15). Studies of MP trafficking have been aided by use of the green fluorescent protein (GFP) (9, 16), showing that MPs accumulate in PDs and interact with the cytoskeleton, a possible route for PD targeting (6, 17–19). GFP expression also provided unexpected insights into the regulation of PD SEL during development. Estimates of SEL based on dye-injection studies vary from one to a few kilodaltons in most cell types (20–22). However, in plants where the sucrose transporter (AtSuc2) promoter drives companion cell-specific expression of GFP, fluorescence spreads through the phloem into sink leaves and unloads into mesophyll and epidermal cells (10, 23). The use of GFP fusions indicates that the SEL for nontargeted movement in leaves is up to 30–55 kDa (10, 24, 25). Moreover, the free movement of GFP is not restricted to sink tissues; it also moves freely between Arabidopsis leaf epidermal cells regardless of developmental stage (24). These studies indicate that the SEL is higher than once thought; however, the compact structure of GFP (26) makes these findings difficult to relate to other proteins. Other factors, such as subcellular localization, may also determine whether a protein can traffic (25). These studies suggest that protein trafficking is widespread, implying its importance in physiology and development.

Dye-injection and -loading studies reveal dynamic developmental regulation of PD communication (27–29). Whether similar regulation also exists for trafficking of regulatory proteins or RNAs is not known. However, many developmental genes act nonautonomously, including members of the knotted related (KNOX) class of homeobox genes that were first characterized by dominant mutations affecting leaf cell fate (30). Qualitative differences in the nonautonomy of these different genes imply specific regulation of signaling (5). In the case of Kn1, we suggested a mechanism for its nonautonomy when we used injections of fluorescent-labeled KN1 protein to show that it has the ability to traffic between cells, to gate PD, and to specifically traffic its mRNA (31). It appeared that movement was a specific and regulated property of KN1, because we identified a mutant, KN1(M6), which was unable to traffic. kn1 and its Arabidopsis homologue, SHOOTMERISTEMLESS (STM), are normally expressed in the SAM, where they function in meristem initiation and/or maintenance (32–36). It is not known whether these proteins traffic in the SAM, although localization studies suggest that this may be true for KN1 (37). We also do not know whether KN1 expressed in vivo is able to traffic between cell layers in the leaf, and the mechanism of KN1 trafficking is poorly understood. Here we show that a GFP–KN1 fusion can traffic within and between cell layers in the leaf; however, the M6 mutant of KN1 and a GFP–yellow fluorescent protein sequence

www.pnas.org/cgi/doi/10.1073/pnas.052484099

PNAS | March 19, 2002 | vol. 99 | no. 6 | 4103–4108

This paper was submitted directly (Track II) to the PNAS office.

Abbreviations: PD, plasmodesma; SEL, size-exclusion limit; ER, endoplasmic reticulum; GFP, green fluorescent protein; YFP, yellow fluorescent protein; MP, movement protein; SAM, shoot apical meristem; UAS, upstream activation sequence.

1J.Y.K. and Z.Y. contributed equally to this work.
2Present address: Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, MA 02115.
3To whom reprint requests should be addressed. E-mail: jackson@chil.org.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. §1734 solely to indicate this fact.
protein (YFP) fusion do not traffic. GFP~KN1 could also traffic between cell layers in the SAM. Our results support the hypothesis that KN1 trafficking in vivo may be part of its normal function.

**Methods**

**DNA Constructs.** For construction of GFP fusions, restriction sites were introduced at the start or stop codon of *knl* and GFP by using oligonucleotide primers and proofreading PCR (Pfu polymerase, Stratagene). PCR products were sequenced to ensure fidelity. Long primers were used to create the 10-alanine linkers at the N or C terminus of GFP, with appropriate restriction sites for ligation of other coding sequences. Constructs were assembled in the pRTL2 vector (38) for bombardment assays. The GFP-KN1 (M6) and GFP-YFP constructs were made by replacing the KN1 sequence with the respective sequence, amplified by PCR to introduce restriction sites. For generation of transgenic plants, inserts were excised and cloned into a PCAMIBA binary vector, introduced into *Agrobacterium tumefaciens* strain GV3101 and transformed into *Arabidopsis* by the floral dip procedure (39). For generation of promoter (pSCR, pUAS) constructs, the inserts were cloned downstream of the respective promoter sequences (40, 41) in PCAMIBA binary vectors. For the *AitRbcS-2b* construct, 1.7 kb upstream of the translation start site was amplified by the PCR from Ler genomic DNA and cloned upstream of GFP~KN1 in a PCAMIBA vector.

**Western Blots.** Protein gels and Western blots were as described (42). For GFP detection, an anti-GFP monoclonal antibody (Zymed) was used at 1:1,000 dilution.

**Microprojectile Bombardment.** *Arabidopsis* plants were grown in short days (8 h light, 16 h dark) for 5–7 weeks and bombarded at a pressure of 450 psi (3.1 MPa) by using a helium biolistic device (Bio-Rad PDS-1000) with 0.15 μg of DNA coated onto 1.5 mg of 1-μm gold particles, according to the manufacturer's instructions (Bio-Rad), and ref. 43 [similar results were obtained with the Bio-Rad Helios Gene Gun (not shown)]. Both Ler and Col ecotypes were used with similar results. Plants were returned to short days and observed 2 days later for GFP fluorescence and trafficking, and leaves 10–25 mm long were excised for imaging. Onion bulb scale pieces (25 × 25 mm) were bombarded at 900 psi (6.2 MPa) and kept in a humid chamber for 2 days, and the epidermis was peeled and mounted in water for imaging.

**Microscopy.** Bombarded tissues and transgenic plants were first screened at low magnification by using a fluorescence dissecting microscope (Leica, Deerfield, IL), and tissues were dissected and mounted in water for viewing in the confocal microscope (Zeiss LSM510). For observation of upstream activation sequence (UAS) lines, free-hand cross sections of leaves were cut by using a double-sided razor blade and mounted in water. Images were scanned electronically in the confocal or with a digital camera (Spot RT, Diagnostic Instruments) on the dissecting microscope.

**Results**

To find an optimal fusion, we made both N- and C-terminal fusions of KN1 to mGFP6, a plant-optimized GFP (gift from J. Haseloff, Medical Research Council, Cambridge, U.K.). In each, we introduced a 10-alanine linker between GFP and KN1 to improve stability and folding (44). We represent this linker as “…-…” i.e., GFP~KN1 or KN1~GFP. We tested the phenotypic effect of overexpression of these fusions in *Arabidopsis*. Overexpression of KN1~GFP (not shown) or of GFP~KN1 (Fig. 1) resulted in lobed leaves, stunted growth, and abnormal flowers. These phenotypes resembled overexpression of KN1 not fused to GFP (not shown) or of KNAT1 (45). GFP~KN1 overexpression also resulted in ectopic shoots on the adaxial leaf surface (Fig. 1C) and gave consistently stronger phenotypes than KN1-GFP (>50 independent lines per construct). Furthermore, transient overexpression of KN1~GFP often led to the production of large fluorescent aggregates (not shown), suggesting that this conformation was not ideal. We therefore used GFP~KN1 in further experiments.

To verify that the GFP~KN1 fusion was stable, Western blots of extracts from leaves expressing GFP or GFP~KN1 were probed with antibodies against KN1 or GFP (Fig. 1D). In each, we detected the expected size products, and there was no evidence of GFP~KN1 degradation, suggesting that we detected movement of green fluorescence in GFP~KN1-expressing plants, it should represent the trafficking of GFP~KN1 and not the nonspecific movement of a smaller degradation product.

We next asked whether GFP~KN1 could traffic cell to cell after transient expression. We also made constructs to express mGFP6, a nontargeted GFP, which we expected would display extensive movement (10, 24, 25), GFP~YFP, a 55-kDa fusion that should be restricted in movement (10, 24, 25), and GFP~MP, a 58-kDa fusion to the MP of turnip vein clearing tobamovirus, which infects *Arabidopsis* (46). In other plants, tobamovirus MP-GFP fusions traffic and localize to PDs (9, 25, 47, 48).

The DNA constructs were introduced by microprojectile bombardment and were scored after 2 days; individual events were defined as isolated fluorescent cells or as clusters of fluorescent cells if movement had occurred. We did not score areas that had a high density of transformed cells, because they may have sustained more damage during bombardment, and
because there could be clusters of fluorescent cells caused by transformation of adjacent cells rather than by cell-to-cell movement. For the same reason, we also did not score the rare events that had adjacent cells of equal fluorescence intensity.

We observed extensive intercellular trafficking of free GFP and GFP–MP in 72 and 59% of events, respectively, generating clusters of between 2 and 30 fluorescent cells in Arabidopsis leaf epidermis (Fig. 2, Table 1). In contrast, GFP–YFP appeared to be mostly cell autonomous, as only 1.6% of events showed any evidence of movement and were always restricted to a group of two cells. These results agree with previous studies (9–11, 25). We observed trafficking of GFP–KN1 in 14.6% of events, to fluorescent clusters of up to four cells. GFP–KN1 localized to the nuclei and cytoplasm of the bombarded cell and adjacent cells into which trafficking had occurred in both Arabidopsis and onion (Fig. 2 E and F). Therefore, both the frequency and range of GFP–KN1 movement were less than that of GFP–MP or GFP but were significantly higher than the smaller GFP–YFP (Table 1).

To test whether GFP–KN1 could traffic between cell layers in the Arabidopsis leaf, we directed tissue-specific expression by using a Gal4 enhancer trap system (41, 49). In this system, a genomic enhancer drives expression of the Gal4–VP16 chimeric transcription factor, which can transactivate a reporter through a Gal4 enhancer trap system (41, 49). In this system, a transposon cassette was driven by a Gal4 enhancer trap system (41, 49). GFP fluorescence is green, and background chlorophyll autofluorescence is red. (Bars in A, B, F = 100 μm; C–E = 50 μm.)

Table 1. Arabidopsis bombardment results

<table>
<thead>
<tr>
<th>Expressed protein</th>
<th>Molecular mass, kDa</th>
<th>% events with movement</th>
<th>Range of no. of cells/cluster</th>
<th>No. of events counted (no. of experiments)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GFP</td>
<td>27</td>
<td>72%</td>
<td>2–30</td>
<td>204 (2)</td>
</tr>
<tr>
<td>GFP–YFP</td>
<td>55</td>
<td>1.6%</td>
<td>2</td>
<td>640 (4)</td>
</tr>
<tr>
<td>GFP–MP</td>
<td>58</td>
<td>59%</td>
<td>2–21</td>
<td>205 (2)</td>
</tr>
<tr>
<td>GFP–KN1</td>
<td>68</td>
<td>14.6%</td>
<td>2–4</td>
<td>1,312 (5)</td>
</tr>
</tbody>
</table>

The table shows the percent of bombardment events where cell-to-cell movement was apparent and the range of the number of fluorescent cells in individual movement events. Data were collected from studies where positive (GFP or GFP–MP) and negative (GFP–YFP) controls were bombarded in the same experiment as GFP–KN1.
vegetative SAM, predominantly in the L1 layer (40). pSCR-GFP–KN1 transgenic plants had a relatively normal shoot architecture but with reduced lobed leaves, presumably because pSCR drives expression in developing leaf primordia in addition to the SAM (40) [Fig. 4 A and B]. pSCR-GFP–KN1 seedlings (Right). We imaged GFP fluorescence in the inflorescence SAM, which is easier to dissect than vegetative SAMs. In the inflorescence SAM, pSCR directed expression of the cell-autonomous mGFP5ER reporter in both the L1 and L2 layers (Fig. 4 B and C). In contrast, fluorescence of GFP–KN1 was detected in underlying L3 layers, in addition to L1 and L2 (Fig. 4 F and G). Similar localization of GFP–KN1 was found in five independent transgenic lines (not shown). The images were a little diffuse, because the GFP–KN1 signal was weak, and we had to increase the confocal pinhole size to allow sufficient light collection. Therefore, it was difficult to determine whether GFP–KN1 localization in SAM cells was nuclear; however, we were sure of the specific presence of GFP–KN1 in the L3, as we imaged the mGFP5ER lines using the same pinhole setting and did not detect any L3 fluorescence. To determine whether cell-to-cell movement in the SAM was a specific property of GFP–KN1, we also expressed another transcription factor, Gal4-VP16, in the L1 layer of the inflorescence meristem using the AINL1 promoter (51). The distribution of Gal4-VP16 protein was monitored in a plant carrying the cell-autonomous mGFP5ER transgene under the control of UAS sequences. In these plants, GFP fluorescence was restricted to the L1 layer of the inflorescence SAM and primordia, indicating that the 25-kDa Gal4-VP16 protein was cell autonomous. In summary, GFP–KN1, but not the smaller Gal4-VP16 fusion, was able to traffic between cell layers in the inflorescence SAM, suggesting that in the meristem, as in the leaf, KN1 contains specific targeting signal(s) for cell-to-cell trafficking.

In summary, a GFP–KN1 fusion that is biologically active when overexpressed was able to traffic to cell to cell in bombardment assays or when expressed using tissue-specific promoters in the leaf or the SAM. GFP–KN1 was present in both the cytoplasm and the nucleus in cells in which it was expressed and in those cells into which it trafficked, and also displayed a punctate cell wall localization. Neither GFP–YFP nor GFP–KN1(M6) control fusions could traffic between cell layers in the leaf. Our results support the hypothesis that plasmodesmal trafficking of KN1 between cell layers in the leaf is responsible for the nonautonomous action of the dominant Kn1 allele in maize, and that cell-to-cell trafficking may be important for the normal function of KN1 in the SAM.

Discussion

Cell-to-cell communication is critical for cell fate determination in plants and may occur by trafficking of regulatory proteins through PD. In maize, noncell autonomous action of a dominant Kn1 allele leads to alterations in leaf cell fate, and in support of the hypothesis that KN1 itself was the signal, we previously showed that this protein could traffic between mesophyll cells in microinjection assays (31). However, those studies did not properly assess the range of trafficking, whether it could occur between different cell types, or whether KN1 could traffic in the SAM, where it functions. Here we show that a GFP fusion of KN1 was able to traffic between cells in the leaf and SAM. Overexpression of GFP–KN1 led to similar developmental phenotypes observed from overexpression of other KNOX genes in Arabidopsis, indicating that this fusion was biologically active.

Cell-to-cell trafficking of GFP–KN1 after bombardment occurred at a relatively low frequency compared with that of GFP–MP, and the range of movement was less, producing clusters of two to four fluorescent cells in approximately 15% of events, compared with clusters of 2–21 cells for GFP–MP in 60% of events. Even nontargeted GFP, which is thought to traffic nonspecifically through PDs, did so in only 72% of bombardment events, indicating that the basal SEL is not the same in all leaf epidermal cells (25). We are confident that the movement of...
GFP–KN1 was significant, because the frequency of movement was almost 10 times higher than the background level reported by the nontargeted GFP–YFP fusion. The low frequency and range of trafficking of GFP–KN1 within the epidermal layer suggest that trafficking is tightly regulated and that the competence of PDs for perception of a hypothetical KN1 trafficking signal was different in different epidermal cells. A similar hypothesis of cell-to-cell variation in PD characteristics was previously proposed to explain why GFP–MP or GFP did not traffic in all bombardment events (11). An alternative explanation for the relatively infrequent trafficking of GFP–KN1 is that the fusion of GFP might have impaired its ability to traffic, although this is unlikely because GFP–KN1 was able to traffic efficiently in stable transgenic plants.

Movement of GFP–KN1 between cell layers in the leaf occurred more readily, from the cells surrounding the vascular tissue through three to four layers of mesophyll cells to the epidermis. Here, GFP–KN1 localized to the nucleus, suggesting that KN1 trafficking is likely to be biologically relevant. Trafficking appeared to be a specific property of GFP–KN1, because the smaller GFP–YFP fusion was unable to traffic in this assay. Furthermore, the M6 mutant of KN1, which was unable to traffic in microinjection assays, also behaved cell autonomously when expressed as a GFP fusion. These controls indicate that trafficking of the GFP–KN1 fusion is likely to be directed by a specific signal in KN1, rather than by a nonspecific mechanism such as diffusion. The reason for the superior trafficking of GFP–KN1 in transgenic plants compared with the bombardment assays could simply be related to time available for trafficking; the bombardments were scored after 2 days, whereas transgenic plants were grown for several weeks before imaging. Alternatively, the GFP–KN1 fusion may traffic more readily between cell layers than in a lateral direction within a specific (epidermal) layer. This hypothesis implies that PDs connecting cells within a layer are functionally different from those between cell layers, which may be true, because the PDs within a layer can be primary, as they are produced during cytokinesis, whereas those that connect cells in adjacent layers are secondarily formed through preexisting cell walls. An indication that KN1 signaling, and presumably movement, may occur more readily between cell layers than within a layer came from the Kn1 mosaic studies, where Kn1 acted nonautonomously between cell layers in the leaf but was relatively autonomous in a lateral direction (52).

Whether this was because of differences in KN1 signaling between layers compared to within a layer, or because of the resolution of the mosaic analysis is not clear; movement through four cells in a maize leaf primordium is sufficient to reach from the provascular tissue to the epidermis, whereas movement through only four cells in a lateral direction could lead to the impression of lateral autonomy. Our data on trafficking of GFP–KN1 suggest, however, that there are indeed real differences in trafficking between cell layers compared with laterally within a layer.

GFP–KN1 was also able to traffic between cell layers in the SAM. When expressed in L1 and L2, GFP–KN1 moved through at least two layers of L3 cells, suggesting that KN1 can move over a range of several cells in the meristem. We previously proposed that KN1 protein traffics from L2 to L1 in the maize SAM, whereas those that connect cells in adjacent layers are secondarily formed through preexisting cell walls. An indication that KN1 signaling, and presumably movement, may occur more readily between cell layers than within a layer came from the Kn1 mosaic studies, where Kn1 acted nonautonomously between cell layers in the leaf but was relatively autonomous in a lateral direction (52).

Whether this was because of differences in KN1 signaling between layers compared to within a layer, or because of the resolution of the mosaic analysis is not clear; movement through four cells in a maize leaf primordium is sufficient to reach from the provascular tissue to the epidermis, whereas movement through only four cells in a lateral direction could lead to the impression of lateral autonomy. Our data on trafficking of GFP–KN1 suggest, however, that there are indeed real differences in trafficking between cell layers compared with laterally within a layer.
How widespread is protein movement in the SAM? The basal SEL for PD in immature leaves is around 30–55 kDa, if measured by using nonspecific GFP fusions (10, 24, 25). Might then all proteins below this molecular mass move freely in the SAM, which is also a pool of undifferentiated cells? Our data suggest that this is not the case, because the 25-kDa Ga4-GFP16 protein appears to be autonomous when expressed in the L1 (results shown here) or L3 layers of the SAM or in various leaf cell types (J.Y.K., M.C., and D.J., unpublished work). Selectivity of trafficking was also observed in the case of the floral homeotic proteins APETALA1 and LEAFY; whereas LEAFY protein was found to act nonautonomously and to traffic from the L1 to L2 and L3 cells, there was only limited localization of the FLORAL ORCHARD protein in cell fate specification, yet the related SCARECROW protein is cell autonomous (55). Therefore, movement is likely to be an active process regulated by trafficking signals in specific proteins. The findings that a specific mutation, KN1(M6), or phagoc changes related to KN1, can block KN1 movement support this hypothesis (31, 56).

Earlier studies also indicated that KN1 could selectively traffic its mRNA, and we suggested that this function could amplify the intercellular KN1 signal. However, this finding was contradictory to the observation that KN1 protein, but not mRNA, is detected in the L1 layer of the SAM. Either the amount of mRNA movement is below the limits of detection by standard in situ hybridization procedures, or the movement of the mRNA may occur between cells in the L2 but not from L2 to L1. We did not assay for GFP–KN1 mRNA movement, so we do not know whether it moves in association with GFP–KN1. However, a recent report showed that a mutant fusion transcript between LeT6, a tomato KN1 related gene, and PYROPHOSPHATE-DEPENDENT PHOSPHOKINASE is able to move along distances in the phloem. Remarkably, the fusion transcript can exit from the phloem and enter the SAM of the scion, and its presence is associated with altered leaf morphology (57). Therefore, long-distance movement of this fusion transcript is associated with alterations in leaf development. The relative importance of protein and mRNA movement for developmental control remains to be seen, although in the case of KN1, the protein likely provides the specificity for movement of its mRNA (51).

What might be the role of KN1 movement in the SAM? We have suggested that in maize, it could serve as a signal to coordinate the development of the L1 layer in response to that of the L2. However, in Arabidopsis, the kn1 homolog STM, which appears to be the closest related to kn1 by its loss of function phenotype and expression pattern, shows no layer-specific differences in its mRNA and protein localization (32, 58). It remains to be seen whether STM traffics and whether this is important for its function. If STM does traffic, the mechanism may be differentially regulated in the two species, and STM movement could play a more general role in communication and coordination of cell fate in the SAM domain or may be a redundant mechanism to ensure all cells adopt a SAM fate, similar to the proposed function of LEAFY trafficking (54). An alternative function for movement could be to generate a gradient of protein concentration at the KN1 or STM expression boundary, which could activate different boundary-specific genes in a mechanism analogous to the patterning of the Drosophila syncytial embryo by gradients of homeodomain proteins (59). Therefore, there may be regulation not only at the level of which proteins can traffic but also apparent in regulated spatial domains for movement. Such domains exist for dye movement in the SAM, suggesting that this level of regulation is likely (28, 29). We are currently investigating the trafficking of GFP–KN1 and other fusion proteins in specific regions of the SAM to address this possibility.

We thank Jim Haseloff for mGFP6 and Ga4 constructs and for making available the Ga4 enhancer trap lines, Philip Benfey and Jocelyn Malamy (New York University) for the SCR promoter clone and TAC-GaFP16 seed, and Vitaliy Citovsky [State University of New York (Stony Brook)] for the turnip vein clearing tobamovirus MP clone. Funding was provided by the National Science Foundation Integrative Plant Biology grant 9727959 and Cold Spring Harbor Laboratory institutional funds. M.C. is the recipient of a Watson School of Biological Sciences William Miller Fellowship and was a Beckman Graduate Student for part of these studies. Z.K. was funded by the Cold Spring Harbor Laboratory Undergraduate Research Program.

4. Ding, B., Itaya, A. & Woo, Y. M. (1999) in International Review of Cytology (D. J., unpublished work). Selectivity of trafficking was also observed in the case of the floral homeotic proteins APETALA1 and LEAFY; whereas LEAFY protein was found to act nonautonomously and to traffic from the L1 to L2 and L3 cells, there was only limited localization of the FLORAL ORCHARD protein in cell fate specification, yet the related SCARECROW protein is cell autonomous (55). Therefore, movement is likely to be an active process regulated by trafficking signals in specific proteins. The findings that a specific mutation, KN1(M6), or phagoc changes related to KN1, can block KN1 movement support this hypothesis (31, 56).

Earlier studies also indicated that KN1 could selectively traffic its mRNA, and we suggested that this function could amplify the intercellular KN1 signal. However, this finding was contradictory to the observation that KN1 protein, but not mRNA, is detected in the L1 layer of the SAM. Either the amount of mRNA movement is below the limits of detection by standard in situ hybridization procedures, or the movement of the mRNA may occur between cells in the L2 but not from L2 to L1. We did not assay for GFP–KN1 mRNA movement, so we do not know whether it moves in association with GFP–KN1. However, a recent report showed that a mutant fusion transcript between LeT6, a tomato KN1 related gene, and PYROPHOSPHATE-DEPENDENT PHOSPHOKINASE is able to move along distances in the phloem. Remarkably, the fusion transcript can exit from the phloem and enter the SAM of the scion, and its presence is associated with altered leaf morphology (57). Therefore, long-distance movement of this fusion transcript is associated with alterations in leaf development. The relative importance of protein and mRNA movement for developmental control remains to be seen, although in the case of KN1, the protein likely provides the specificity for movement of its mRNA (51).

What might be the role of KN1 movement in the SAM? We have suggested that in maize, it could serve as a signal to coordinate the development of the L1 layer in response to that of the L2. However, in Arabidopsis, the kn1 homolog STM, which appears to be the closest related to kn1 by its loss of function phenotype and expression pattern, shows no layer-specific differences in its mRNA and protein localization (32, 58). It remains to be seen whether STM traffics and whether this is important for its function. If STM does traffic, the mechanism may be differentially regulated in the two species, and STM movement could play a more general role in communication and coordination of cell fate in the SAM domain or may be a redundant mechanism to ensure all cells adopt a SAM fate, similar to the proposed function of LEAFY trafficking (54). An alternative function for movement could be to generate a gradient of protein concentration at the KN1 or STM expression boundary, which could activate different boundary-specific genes in a mechanism analogous to the patterning of the Drosophila syncytial embryo by gradients of homeodomain proteins (59). Therefore, there may be regulation not only at the level of which proteins can traffic but also apparent in regulated spatial domains for movement. Such domains exist for dye movement in the SAM, suggesting that this level of regulation is likely (28, 29). We are currently investigating the trafficking of GFP–KN1 and other fusion proteins in specific regions of the SAM to address this possibility.

We thank Jim Haseloff for mGFP6 and Ga4 constructs and for making available the Ga4 enhancer trap lines, Philip Benfey and Jocelyn Malamy (New York University) for the SCR promoter clone and TAC-GaFP16 seed, and Vitaliy Citovsky [State University of New York (Stony Brook)] for the turnip vein clearing tobamovirus MP clone. Funding was provided by the National Science Foundation Integrative Plant Biology grant 9727959 and Cold Spring Harbor Laboratory institutional funds. M.C. is the recipient of a Watson School of Biological Sciences William Miller Fellowship and was a Beckman Graduate Student for part of these studies. Z.K. was funded by the Cold Spring Harbor Laboratory Undergraduate Research Program.