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†¶

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

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Dominant mutations in the maize homeobox 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.

GFP | plasmodesmata | homeodomain | internal ribosome entry sequence | KNOX

The 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 apoplasm, 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

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Abbreviations: PD, plasmodesmata; 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.

†J.Y.K. and Z.Y. contributed equally to this work.

§Present address: Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, MA 02115.

¶To whom reprint requests should be addressed. E-mail: jacksond@cshl.org.

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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 *kn1* 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 pCAMBIA binary vector, introduced into *Agrobacterium* strain GV3101 and transformed into *Arabidopsis* by the floral dip procedure (39). For generation of promoter (p*SCR*, p*UAS*) constructs, the inserts were cloned downstream of the respective promoter sequences (40, 41) in pCAMBIA binary vectors. For the *AtRbcS-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 pCAMBIA 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 $\times$ 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

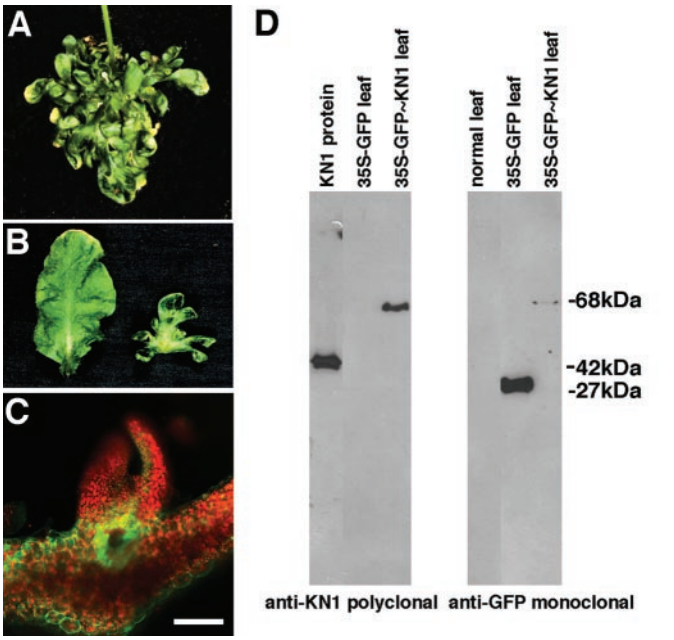

Fig. 1. Overexpression of a GFP~KN1 fusion in *Arabidopsis*. A 35S-GFP~KN1 plant (NoO ecotype) shows the stunted lobed leaf phenotype (*A*). Isolated leaves are in *B*, normal rosette leaf (*Left*), lobed 35S-GFP~KN1 leaf (*Right*). GFP~KN1 overexpressers produce ectopic shoots on the adaxial leaf surface; a confocal fluorescence image of an ectopic shoot on a leaf expressing GFP~KN1 under the *Arabidopsis RbcS-2b* promoter is shown in *C*. GFP~KN1 fluorescence is green, chlorophyll autofluorescence is red. (Bar = 100 μm.) (*D*) Western blots of extracts from leaves of GFP or GFP~KN1 overexpressers. (*Left*) Probed with anti-KN1 polyclonal antibody (42) and a band of the predicted size (42 kDa) is found in extracts from *E. coli* expressing KN1. Extracts from leaves of GFP~KN1 overexpressers have a crossreacting band of the expected size (68 kDa). Extracts from leaves of GFP or GFP~KN1 overexpressing seedlings probed with anti-GFP monoclonal antibody reveal bands of 27 and 68 kDa, respectively, as expected.

also resulted in ectopic shoots on the adaxial leaf surface (Fig. 1*C*) 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. 1*D*). In each, we detected the expected size products, and there was no evidence of GFP~KN1 degradation, suggesting that if 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

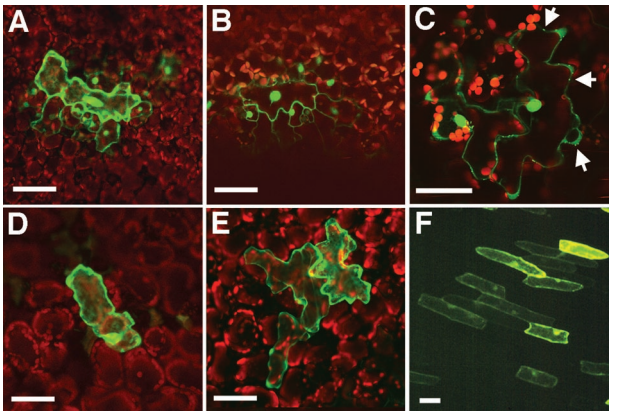

Fig. 2. Intercellular trafficking of GFP fusion proteins after microprojectile bombardment. Nontargeted GFP shows extensive cell-to-cell movement to a cluster of approximately 20 epidermal cells and is present throughout the cytoplasm and nucleoplasm (*A*). The GFP~MP fusion shows similar extensive cell-to-cell movement (*B* and *C*). The close-up (*C*) shows fluorescence in a punctate cell wall pattern, presumably corresponding to clusters of PDs. The GFP~YFP fusion (*D*) is cell autonomous, as fluorescence is restricted to single cells. GFP~KN1 is present in small clusters of cells in *Arabidopsis* (*E*) and onion (*F*); nuclear staining in *Arabidopsis* is not visible in this confocal section but is evident in the onion cells. All images are taken in the confocal microscope. GFP fluorescence is green, and background chlorophyll autofluorescence is red. (Bars in *A*, *B*, *F* = 100 μm; *C*–*E* = 50 μm.)

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 fusion (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 Gal4 UAS. We used the J2111 enhancer trap line, which drives expression of the cell-autonomous ER localized mGFP5ER reporter (50) in cells surrounding the vascular tissue (Fig. 3 *C* and *D*). Kanamycin-resistant J2111 plants were transformed with a UAS-GFP~KN1 construct carrying hygromycin resistance, and double transformants were selected on kanamycin plus hygromycin. Of 27 transformants, 5 had a KN1 overexpression phenotype (Fig. 3 *G*–*I*), indicating that UAS-GFP~KN1 was transactivated in these plants. As a control, we made approximately 50 transformants of the UAS-GFP~KN1 construct into wild-type *Arabidopsis*, and none had overexpression phenotypes or GFP fluorescence (not shown), so the expression of GFP~KN1 in the J2111 lines was because of transactivation by the J2111 Gal4 driver. Genetic crossing of UAS-GFP~KN1 plants to J2111 plants also gave progeny in which GFP~KN1 was activated (not shown).

Imaging of leaves from J2111 seedlings revealed GFP fluorescence in cells surrounding the vascular tissue, which we presumed to be bundle sheath (Fig. 3 *C* and *D*). No green fluorescence was detected in mesophyll or epidermal cells. In contrast, leaves from J2111 seedlings containing UAS-GFP~KN1 had green fluorescence in addition in mesophyll and epidermal cells (Fig. 3 *I*, *J*, and *L*). In confocal images of epidermal cells, we detected GFP~KN1 fluorescence in nuclei as well as in bright spots in the cell wall (arrows, Fig. 3*L*). The identity of these spots is unknown; however, they are reminiscent of GFP~MP localization and might correspond to plasmodesmal clusters or pit fields (Fig. 2*C*). The spots were not detected in guard cell walls (these cells lack PD) or in epidermal cells expressing nontargeted GFP (Fig. 3*F*). To ask whether the interlayer trafficking was a specific property of KN1, we used two control UAS constructs, UAS-GFP~YFP (Fig. 3*E*) and UAS-GFP~KN1(M6) (Fig. 3*K*) in the J2111 enhancer line. In 26 independent transgenic lines for UAS-GFP~YFP and 86 for UAS-GFP~KN1(M6), we never observed any movement of the fluorescence signal, indicating that the GFP~YFP fusion, which is smaller than GFP~KN1, as well as the M6 mutant of KN1, behaved cell autonomously. To be sure that the UAS construct was expressed, we used a construct in which the GFP fusion coding region was followed by an internal ribosome entry sequence (IRES) from turnip vein clearing tobamovirus (J.Y.K. and D.J., unpublished work), then the β-glucuronidase (GUS) coding sequence. In the J2111 plants carrying the UAS-GFP~YFP [or GFP~KN1(M6)]-IRES-GUS constructs, we detected GUS expression in the same perivascular cells where the J2111 enhancer was expressed, indicating that the control GFP fusions were expressed (see Figs. 5 and 6, which are published as supporting information on the PNAS web site, www.pnas.org). In summary, when expressed in cells surrounding the vascular tissue, GFP~KN1 was able to traffic through several mesophyll cell layers into epidermal cells and became localized to the nucleus and to fluorescent spots in the cell wall of those cells. In contrast, GFP~YFP and GFP~KN1(M6) displayed no intercellular trafficking activity.

We next asked whether GFP~KN1 could traffic in the SAM, where KN1 is normally expressed and functions (36). We used the *SCARECROW* (*SCR*) promoter, which drives expression in the

Table 1. *Arabidopsis* bombardment results

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

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.

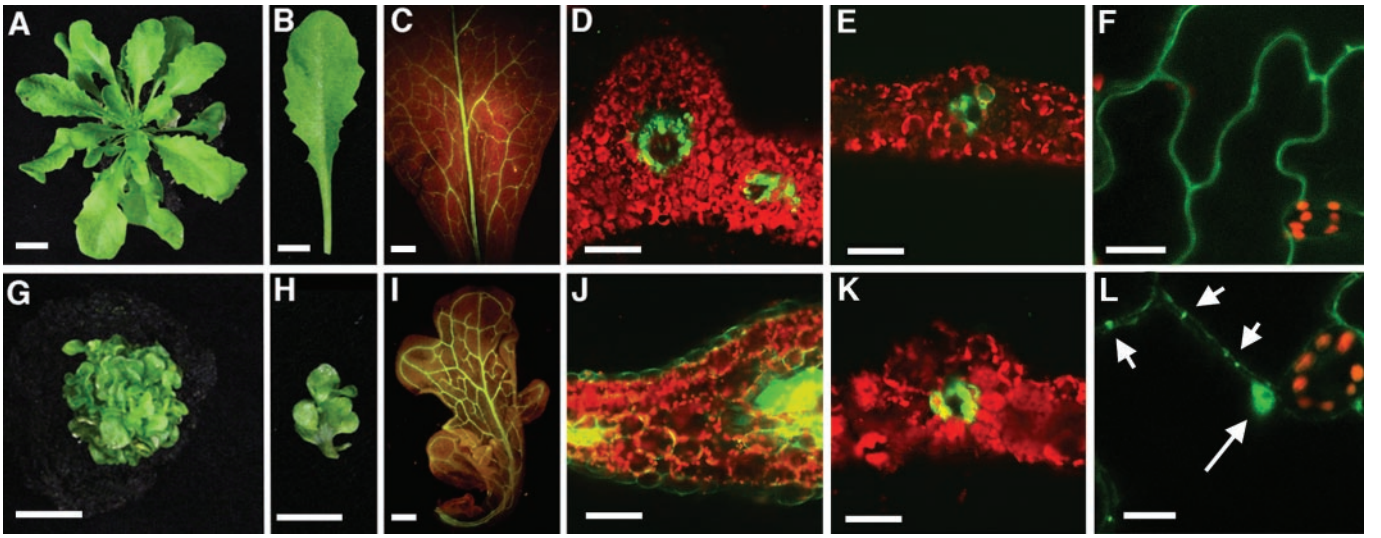

Fig. 3. Interlayer trafficking of GFP∼KN1 in the leaf. The J2111 Gal4 enhancer trap plants are shown in *A–D*; J2111 plants containing the UAS-GFP∼KN1 transgene are in *G–J*. (*A* and *B*) Seedling and rosette leaf of J2111 plants. (*G* and *H*) Seedling and rosette leaf of J2111 plants carrying the UAS-GFP∼KN1 transgene have reduced lobed leaves. Low-magnification fluorescence images of J2111 and J2111/UAS-GFP∼KN1 rosette leaves are shown in *C* and *I*, respectively. Green fluorescence is associated with vascular tissue; the intervascular tissue also appears greenish in the J2111/UAS-GFP∼KN1 leaf. Free-hand cross sections imaged in the confocal microscope show GFP fluorescence specifically in cells surrounding the vascular tissue in the J2111 enhancer trap line (*D*). In J2111/UAS-GFP∼KN1 plants, green fluorescence is also detected in mesophyll and epidermal cells (*J*); mesophyll cells appear paler red compared with *D* because of the combination of red and green. Control J2111 plants containing the UAS-GFP∼YFP (*E*) or UAS-GFP∼KN1(M6) (*K*) do not show any movement of these fusion proteins. Epidermal cells of a plant expressing nontargeted GFP are in *F* and show the expected cytoplasmic fluorescence. Note that the majority of the volume of the epidermal cells is occupied by the vacuole, which does not accumulate green fluorescence. In J2111/UAS-GFP∼KN1 epidermal cells (*L*), fluorescence is visible in the nucleus (long arrow, *L*), cytoplasm, and in spots in the cell wall (short arrows, *L*). Fluorescent spots are not seen in the guard cell walls (these cells contain red autofluorescent chloroplasts). (Bars in *A* and *G* = 10 mm; *B* and *H* = 5 mm; *C* and *I* = 1 mm, *D*, *E*, *J*, and *K* = 100 μm; *F* = 20 μm; *L* = 10 μm.)

vegetative SAM, predominantly in the L1 layer (40). p*SCR*-GFP∼KN1 transgenic plants had a relatively normal shoot architecture but with reduced lobed leaves, presumably because p*SCR* drives expression in developing leaf primordia in addition to the SAM (40) [Fig. 4*A*, normal seedling (*Left*), two p*SCR*-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 *AtML1* 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 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

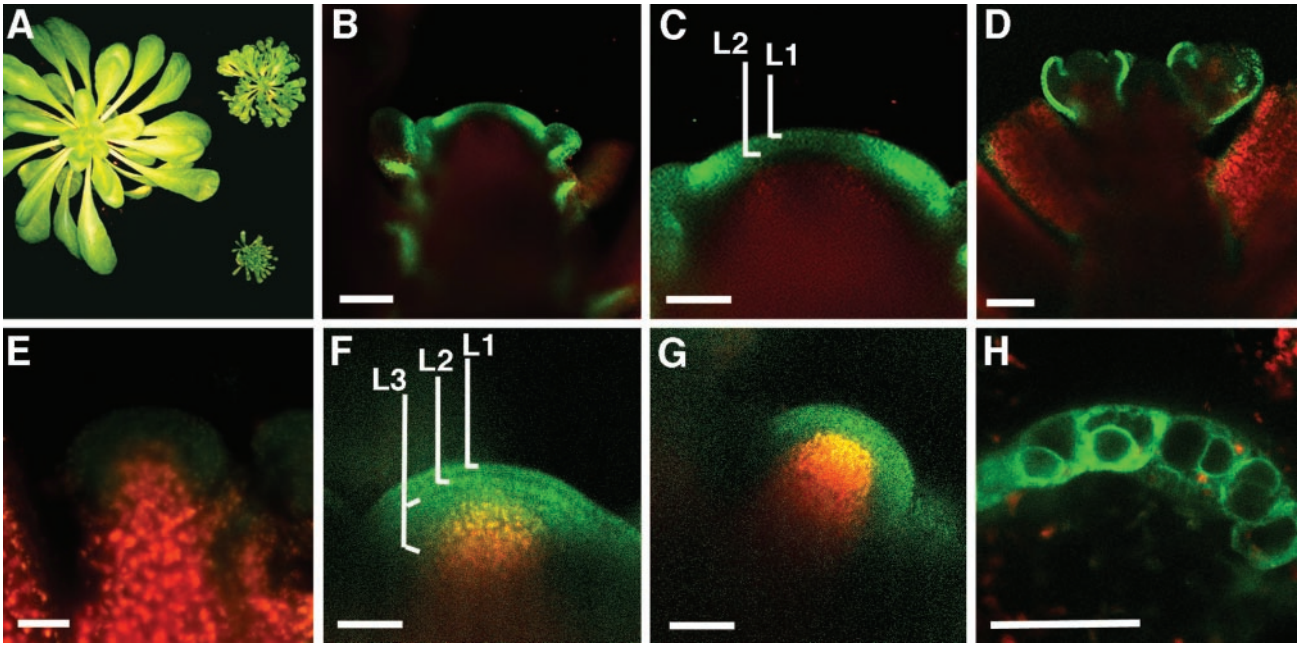

Fig. 4. Interlayer trafficking of GFP~KN1 in the SAM. A normal seedling and two *SCR* promoter-GFP~KN1 seedlings (NoO ecotype) are shown in *A*; p*SCR*-GFP~KN1 plants have a reduced lobed-leaf phenotype. Expression of ER localized GFP by p*SCR* is shown in *B* and *C* and is restricted to L1 and L2 layers of the inflorescence apical meristem and provascular strands in floral primordia. In contrast, GFP~KN1 fluorescence is detected in L3 as well as L1 and L2 (*F*, *G*). Expression of the 25-kDa Gal4-VP16 fusion protein is cell-autonomous in the inflorescence SAM; *D* and *H* show low- and high-magnification images of the inflorescence apex of p*AtML1*-Gal4-VP16/UAS-GFPER plants. Green fluorescence is localized to the ER of L1 cells of primordia and meristems. For comparison, a nontransgenic inflorescence apex imaged under similar conditions is shown in *E*; only the red chlorophyll autofluorescence is visible. (Bars in *B* and *E* = 50 μm; *C*, *F*, and *G* = 25 μm; *D* = 100 μm; *H* = 10 μm.)

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, because KN1 protein, but not mRNA, is detected in L1 cells. Our data therefore suggest that trafficking of KN1 in the SAM may be bidirectional. Our studies also address the mechanism for intercellular trafficking of KN1. Specifically, the punctate cell wall fluorescence supports the hypothesis that intercellular KN1 trafficking is indeed through PD. This need not be an obvious conclusion, because the trafficking of homeodomain proteins between animal cells occurs by specialized secretion and uptake mechanisms (53). In addition, the lateral movement of GFP~KN1 in the epidermis is limited and is significantly less than that of GFP~MP, suggesting that the mechanism for intercellular trafficking of these two proteins differs. Indeed, we saw no evidence for microtubule localization of GFP~KN1, which is suggested to be involved in MP movement (6, 17–19).

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 Gal4-VP16 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 LFY protein was found to act nonautonomously and to traffic from the L1 to L2 and L3 cells, there was only limited nonautonomy of AP1 and no evidence for its movement (54). Similarly, trafficking of SHORTROOT protein is required for its function 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 phage display peptides 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 PHOSPHOFRUCTOKINASE* is able to move long 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 transport 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 (31).

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 *LFY* 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* syncitial 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.

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