Developmental regulation and significance of KNOX protein trafficking in *Arabidopsis*

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SUMMARY

Intercellular communication delivers critical information for position-dependent specification of cell fate. In plants, a novel mechanism for cell-to-cell communication involves the intercellular trafficking of regulatory proteins and mRNAs. The maize *KNOTTED1* (*KN1*) gene acts non cell-autonomously in the maize leaf, and KN1 was the first plant protein shown to traffic cell-to-cell, presumably through plasmodesmata. We have compared the intercellular trafficking of green fluorescent protein (GFP) fusions of KN1 and *Arabidopsis* KN1-related homeobox proteins to that of the viral movement protein from turnip vein clearing tobamovirus. We show that there is specific developmental regulation of GFP~KN1 trafficking. GFP~KN1 was able to traffic from the inner layers of the leaf to the epidermis, but not in the opposite direction, from epidermis to mesophyll. However, GFP or the GFP~movement protein fusion moved readily out of the epidermis. GFP~KN1 was however able to traffic out of the epidermal (L1) layer in the shoot apical

meristem, indicating that KN1 movement out of the L1 was developmentally regulated. GFP~KNAT1/BREVIPEDICELLUS and GFP~SHOOT-MERISTEMLESS fusions could also traffic from the L1 to the L2/L3 layers of the meristem. In a test for the functional significance of trafficking, we showed that L1-specific expression of KN1 or of KNAT1 was able to partially complement the strong *shootmeristemless-11* (*stm-11*) mutant. However, a cell-autonomous GUS fusion to KN1 showed neither trafficking ability nor complementation of *stm-11* when expressed in the L1. These results suggest that the activity of KN1 and related homeobox proteins is maintained following intercellular trafficking, and that trafficking may be required for their normal developmental function.

Key words: Homeodomain, KNOX, Shoot meristem, *knotted1*, GFP, Plasmodesmata, Protein trafficking, *Arabidopsis thaliana*

INTRODUCTION

In the last few years, the intercellular trafficking of regulatory proteins and mRNAs has emerged as a novel mechanism of cell-to-cell communication in plant development (Ding, 1998; Zambryski and Crawford, 2000; Jackson, 2001; Haywood et al., 2002; Wu et al., 2002). Many studies on trafficking of macromolecules relate to virus infection, as some plant viruses move from cell to cell through plasmodesmata (PD). However, the smallest viruses or viral nucleic acids are estimated to be larger than the channel size of PDs (Ding et al., 1992b), and viruses traffic via an active pathway that requires virus encoded movement proteins (MPs) (Gibbs, 1976; Wang et al., 1998). For example, tobacco mosaic virus (TMV) encodes a 30 kDa MP that interacts with PDs to increase their size exclusion limit (SEL), traffics itself and facilitates spread of the virus (Deom et al., 1987; Wolf et al., 1989; Waigmann et al., 1994). The observation of viral MP trafficking prompted speculation that endogenous macromolecules might also traffic through PD (Lucas and Wolf, 1993; Lucas et al., 1993; Maule, 1994).

The use of the green fluorescent protein (GFP) greatly facilitated the development of in vivo trafficking assays. Tissue-specific GFP expression studies also revealed dynamic

regulation of PD SEL. Estimates for PD SEL in mesophyll or trichome cells of mature leaves have been obtained from microinjection experiments, and range from one to a few kDa (Wolf et al., 1989; Waigmann and Zambryski, 1995). However, in immature sink tissues the observation of GFP diffusion suggests that the PD SEL may be as high as 30-50 kDa in these tissues (Imlau et al., 1999; Oparka et al., 1999). Developmental changes in GFP diffusion are correlated with changes in PD structure during leaf development, from simple linear channels to complex branched forms (Oparka et al., 1999). It is also evident that GFP can diffuse cell-to-cell in some mature tissues, depending on the tissue type and species (Crawford and Zambryski, 2000; Itaya et al., 2000; Kim et al., 2002). Developmental modifications to PDs are also relevant to MP localization and trafficking. For example, TMV or cucumber mosaic virus MP has been shown to be targeted to plasmodesmata and trafficked between cells only when the leaf reached a certain developmental stage (Ding et al., 1992; Moore et al., 1992; Itaya et al., 1998). These observations of GFP diffusion and MP trafficking reveal developmental regulation of PD function, suggesting that signaling through PDs is important in plant development. The idea that MP trafficking is related to that of endogenous proteins is also

supported by the fact that trafficking is extremely rapid (Ding, 1998), and that a plant MP-related protein, CmPP16, can traffic itself and mRNA through PDs (Xoconostle-Cazares et al., 1999). However, the extent to which trafficking of endogenous proteins is developmentally regulated is unclear. Endogenous trafficking proteins include phloem proteins (Balachandran et al., 1997) as well as several developmental transcription factors such as LEAFY (LFY), SHORTROOT (SHR) and KNOTTED1 (KN1). The LFY meristem identity protein acts non-autonomously and is able to traffic from the L1 layer to the L2 and L3 meristem cells and to complement a *lfy* mutant (Sessions et al., 2000). In the root, the SHR protein traffics from the stele into the endodermal cell layer, and trafficking appears to be required for its function in cell fate specification (Nakajima et al., 2001). The influence of tissue-specific or developmental signals in trafficking of these proteins is largely unknown.

The first endogenous protein shown to traffic cell-to-cell was the maize homeodomain protein, KN1. Mosaic analysis of a dominant *Kn1* allele showed that it acts non-autonomously during maize leaf development (Hake and Freeling, 1986). Later, in situ hybridization and immunolocalization experiments showed that KN1 protein is detected outside the domain of mRNA expression, suggesting the possibility of KN1 trafficking (Jackson et al., 1994). Microinjection studies of fluorescently labeled KN1 protein showed directly that KN1 has the ability to traffic between mesophyll cells, to increase PD SEL and to specifically transport its mRNA (Lucas et al., 1995). These studies suggested that the KN1 protein itself could be the cell non-autonomous signal, and in support of this hypothesis we showed that a GFP-tagged KN1 fusion is able to traffic in the leaf and shoot apical meristem (SAM) in *Arabidopsis* (Kim et al., 2002). *Arabidopsis* encodes four class I KN1-related homeobox (KNOX) genes (Bharathan et al., 1999; Reiser et al., 2000; Semiarti et al., 2001). The most closely related to KN1 are KNOTTED 1-like homeobox protein 1/BREVIPEDICELLUS (KNAT1/BP) and SHOOTMERISTEMLESS (STM); KNAT1 is thought to be the *Arabidopsis* ortholog of KN1 (Bharathan et al., 1999; Reiser et al., 2000), but STM has closer functional similarity to KN1 on the basis of similar null mutant phenotypes and expression patterns (Long et al., 1996; Vollbrecht et al., 2000). STM and KN1 function in SAM initiation and/or maintenance, and KNAT1/BP is involved in the regulation of inflorescence architecture (Byrne et al., 2002; Douglas et al., 2002; Venglat et al., 2002). The possible involvement of trafficking in the function of the *Arabidopsis* KNOX proteins has not been investigated.

In this report we show a specific difference in inter-layer trafficking in the *Arabidopsis* leaf between GFP~MP and GFP~KN1. Our observations suggest that different mechanisms are involved in the trafficking of these proteins. We also show that GFP~KN1 trafficking is under tissue-specific regulation and can be influenced by developmental stage. In support of a biological function of KNOX protein trafficking, KN1 and the related *Arabidopsis* proteins STM and KNAT1 could traffic out of the L1 layer of the shoot apical meristem, and movement was correlated with the complementation of *stm-11* mutant phenotypes. Based on these findings, we discuss the potential roles for trafficking of KNOX gene products during development.

MATERIALS AND METHODS

Plant material, growth conditions and plant transformation

The *Arabidopsis thaliana* line used for the UAS/GAL4 system was Nossen-*0* (No-*0*), except for the J2111 GAL4 enhancer line, which was in the C24 ecotype. The *stm-11* mutant in Landsberg *erecta* (L*er*) ecotype was a kind gift from Dr Kathy Barton. Plants were grown in the greenhouse on premixed soil (75% sphagnum peat moss, 15% vermiculite, 10% perlite, pH 5.2-6.0; Scotts Customblend Low Plus 1, Scotts Co.) with controlled release fertilizer (12-12-12, Scotts Co.) under 18 hours light provided with fluorescent lamps (150-250 μmole quanta m^{-2} s^{-1}) at 20-24°C. About 4-week-old *Arabidopsis* plants with floral buds were dipped in an *Agrobacterium* strain GV3101 suspension culture in YEP medium (yeast extract 10 g/l, peptone 10 g/l, NaCl 5 g/l, pH 7.5) including 5% sucrose and 0.04% Silwet-77 [modified from the method of Clough and Bent (Clough and Bent, 1998)]. Transgenic seedlings were screened on MS medium (0.43% Murashige and Skoog salt mixture, 2.5 mM 2-[N-morpholino]ethanesulfonic acid, 1× Gamborg's vitamin solution, 0.9% bacto-agar, pH 5.7-5.9) containing 50 μg/ml kanamycin and/or hygromycin in an incubator (22°C, 100 μmole quanta m^{-2} s^{-1}) and then transferred to soil in the greenhouse.

DNA constructs

The p*UAS*-GFP~KN1 construct was described previously (Kim et al., 2002). We used a 10-alanine linker (represented as '~') between GFP and KN1 to improve stability and folding (Doyle and Botstein, 1996; Kim et al., 2002). The MP coding region (GenBank accession no. U03357) of turnip vein clearing tobamovirus (TVCV) that is capable of viral movement/infection in *Arabidopsis* (Lartey et al., 1997) was amplified using proofreading PCR to insert restriction enzyme sites, allowing the replacement of KN1 to produce p*UAS*-GFP~MP. The *RbcS2b*, *LTP1* and *AtML1* gene promoters were amplified by PCR from L*er* genomic DNA and inserted upstream of the GAL4 gene in vector pCambia2300. The primers used were: p*RbcS2b* (5′ primer: GCTGCTAGCTTTACCCTAACTACTCCTTT/3′ primer: GTCGT-CGACCCCGGGTTGTTGTTTCTCTTCTTCTTTT), p*LTP1* (5′ primer: GGGAAGCTTGACCAAAATGATTAACTTGCATTAC/3′ primer: GGGGGATCCATTGATCTCTTAGGTAGTGTTTTATGT) and p*AtML1* (5′ primer: GAGGAATTCTTAATTAACATTGATTCT-GAACTGTACCC/3′ primer: CATGGATCCGGCGCGCCAACCG-GTGGATTCAGGGAG). All other constructs including p*AtML1*-GFP~KN1, p*AtML1*-GFP~STM, p*AtML*-GFP~KNAT1, p*AtML1*-GUS~GFP, p*AtML1*-GUS~KN1, *35S*-GUS~KN1 were prepared by sequential modification (replacement by PCR fragments) of the original GFP~KN1 construct. The PCR fragments were verified by sequencing.

Imaging

T_1 or T_2 *Arabidopsis* plants were grown in long day conditions in an incubator or in the greenhouse. Unless noted otherwise, leaf images were taken from fully expanded leaves. Confocal microscopy was performed as described previously (Kim et al., 2002). For free hand-cut cross sections, tissues were embedded in 4% agarose, cut using a double-sided razor blade and mounted in water. The confocal pinhole was set at 3.0 Airy units (AU) for leaf tissues and at 2.0 AU for apex tissues. Two-photon microscopy and scanning electron microscopy were conducted as described by Oertner et al. (Oertner et al., 2002) and Taguchi-Shiobara et al. (Taguchi-Shiobara et al., 2001) respectively, and whole plants were photographed using a digital camera (Sony).

β-glucuronidase staining and immunolocalization

β-glucuronidase (GUS) staining was performed as described previously (Jefferson, 1987). The stained tissues were fixed in FAA (50% ethanol, 10% formaldehyde, 5% acetic acid) for 1 hour,

dehydrated, cleared and embedded in Paraplast X-tra (Fisher Scientific) (Jackson et al., 1994). Tissue sections (10 μm) were dewaxed and mounted in Cytoseal 60 (Stephens Scientific) mounting medium. Immunolocalization of KN1 was performed as described previously (Lucas et al., 1995).

stm-11 genotyping

Genotyping of The *stm-11* allele was genotyped using a CAPS marker (M. K. Barton, personal communication). Two *stm* primers (5′ primer: GGGCTTGATCAATTCATGGAAGCTTACTGTGAAATGCTCGT-GCAGTATGAG, 3′ primer: CCCTAGTAACAACCATCAAAG) were used to produce a 350 bp fragment from genomic DNA. PCR was performed as follows: 95°C 3 minutes, then 35 cycles (94°C 45 seconds, 60°C 45 seconds, 72°C 1 minute) followed by 72°C for 6 minutes. The *Mwo*I restriction enzyme cuts ~50 bp from the 5′ end of the amplified wild-type fragment, but does not cut the *stm-11* fragment. The enzyme digestion was visualized after running on a 2% agarose gel containing ethidium bromide.

RESULTS

Expression of KN1 in different cell layers of the leaf leads to distinct phenotypes

To determine whether intercellular protein trafficking might be developmentally regulated, for example in different tissue types, we used tissue-specific promoters to express the GFP~KN1 fusion during leaf development. For green tissue (primarily mesophyll and guard cells) we used the rubisco small subunit gene (*RbcS2b*) promoter (Kim et al., 2002). In practical terms for trafficking assays, p*RbcS2b* serves as a mesophyll-specific promoter, because the guard cells are symplastically isolated from other epidermal cells from an early stage of development (Wille and Lucas, 1984; Ding et al., 1997). For epidermis-specific expression we used the *LIPID TRANSFER PROTEIN 1* (*LTP1*) and *MERISTEM LAYER 1* (*AtML1*) promoters (Thoma et al., 1994; Sessions et al., 1999). These promoters were used in the two component GAL4

system to drive the expression of four reporters, an endoplasmic reticulum (ER) localized cell-autonomous GFP (mGFP5-ER), 'free' cytoplasmic GFP, and fusions of GFP to the TVCV MP or to KN1 (Haseloff et al., 1997; Kim et al., 2002). The GAL4-driver and UAS-reporter constructs were sequentially transformed into *Arabidopsis* (Kim et al., 2002).

In all cases, plants expressing GFP, mGFP5-ER (Fig. 1A) or GFP~TVCV MP were indistinguishable from normal plants, for example in leaf shape and plant stature. Plants expressing GFP~KN1, however, showed distinctive developmental phenotypes. When expressing GFP~KN1 in the epidermis using the *LTP1* or the *AtML1* promoters, the plants had a relatively mild phenotype in which the leaves were reduced in size, rumpled and sometimes mildly lobed (Fig. 1B,C,E). The p*AtML1*-GFP~KN1 plants had stronger phenotypes than p*LTP1*-GFP~KN1 plants. The overall stature of these plants upon flowering was normal. In contrast, plants expressing GFP~KN1 from the *RbcS2b* promoter had a more severe phenotype, reminiscent of plants overexpressing KNOX genes using the strong constitutive *35S* promoter (Lincoln et al., 1994; Chuck et al., 1996; Kim et al., 2002). These plants were stunted and had reduced and severely lobed leaves (Fig. 1D,E). Some seedlings had a very severe phenotype where the whole shoot comprising multiple organs, was smaller than a single cotyledon (Fig. 1F); these seedlings also developed ectopic shoots on the leaves, again reminiscent of *35S*-KNOX over-expressors (Lincoln et al., 1994; Chuck et al., 1996) (Fig. 1G). In summary, plants developed distinct phenotypes depending on whether they expressed GFP~KN1 in the epidermis or in the green (predominantly mesophyll) tissues.

To confirm the tissue specificity of the *LTP1*, *AtML1* and *RbcS2b* promoters, we imaged GFP fluorescence in seedlings carrying each of these promoters driving expression of the cell-autonomous mGFP5-ER reporter. Fidelity of the promoters was tested in at least 10 independent plant lines, and representative results are shown in Figs 2 and 3. The *RbcS2b* promoter drove expression as expected in leaf mesophyll and
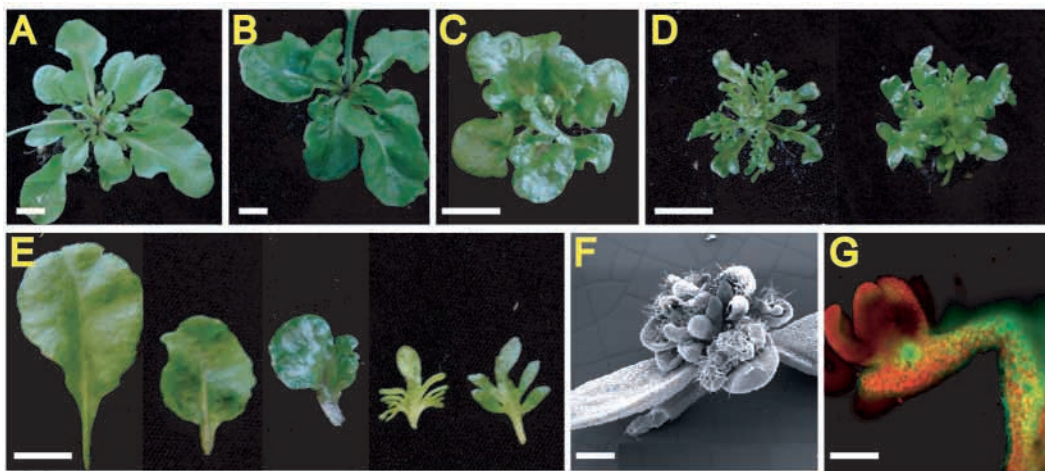

Fig. 1. Tissue-specific expression of a GFP~KN1 fusion produces transgenic plants carrying distinct phenotypes. (A) Plants expressing mGFP5-ER in the mesophyll had a normal phenotype. (B,C) Epidermis-specific expression of GFP~KN1 using p*LTP1* (B) or p*AtML1* (C) resulted a mildly rumpled leaf phenotype. (E) Mesophyll-specific GFP~KN1 expression resulted in lobed leaf phenotypes (D) that were reminiscent of plants overexpressing KNOX genes using the *35S* promoter. (E) Representative leaves of plants shown in A-D are displayed in order. (F,G) Severe p*RbcS2b*-GFP~KN1 seedlings: (F) SEM and (G) a confocal image showing an ectopic shoot on the adaxial side of the leaf (G). Scale bars: 1 cm (A-E), 1 mm (F), 50 μm (G).
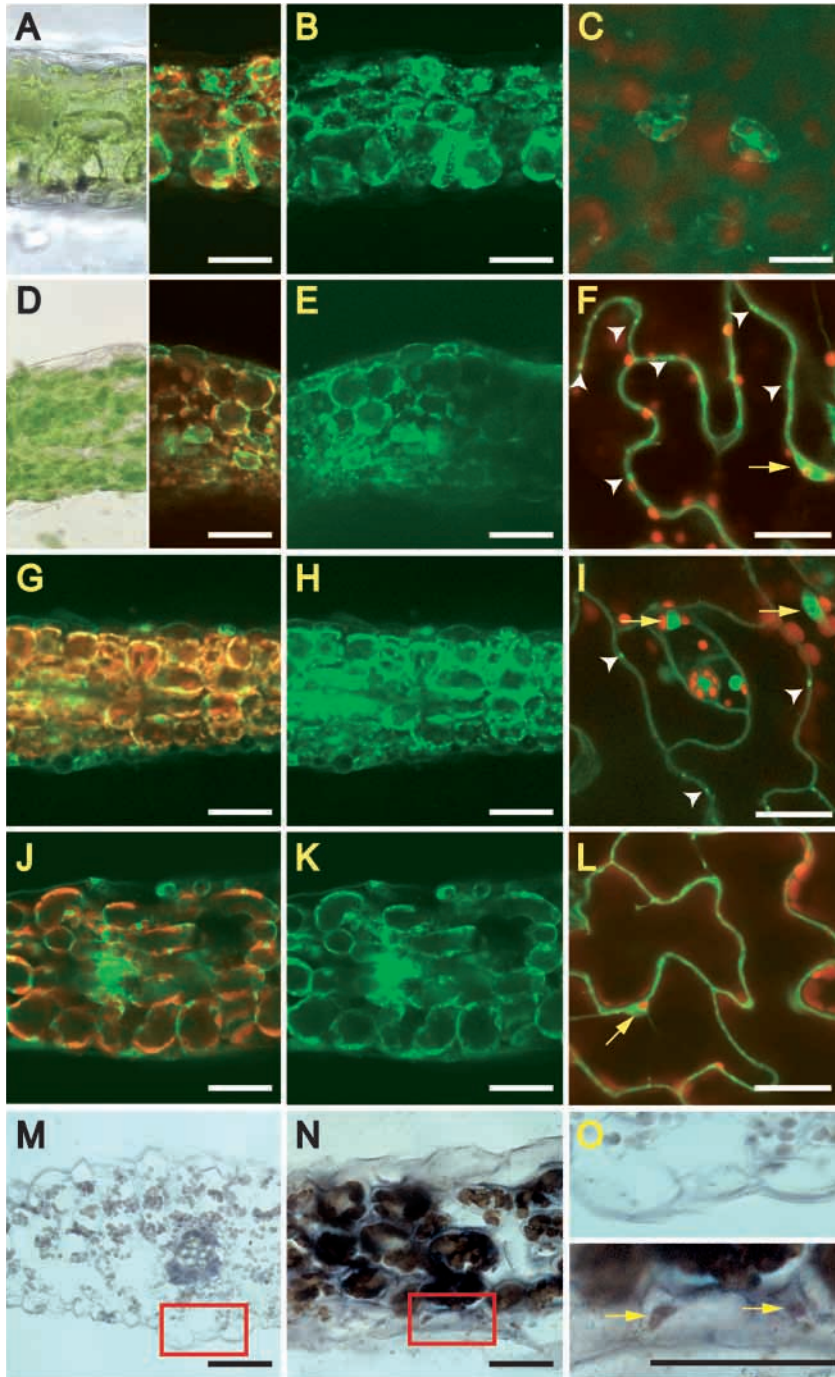
Fig. 2. Protein trafficking from mesophyll to epidermal cells in the *Arabidopsis* leaf. GFP fusion reporters were expressed using the mesophyll-specific p*RbcS2b*- GAL4/ *UAS*-reporter system. The tissue specificity of the promoter is shown by the expression of the cell-autonomous mGFP5-ER reporter.
(A-C) Expanded leaves of transgenic plants expressing mGFP5-ER showed GFP fluorescence in mesophyll and epidermal guard cells, but not in epidermal pavement cells. (D-L) In contrast, the presence of GFP~KN1 (D-F), GFP~TVCV MP (G-I) and free GFP (J-L) under the same promoter was detected in all epidermal and sub-epidermal cells. (F) GFP~KN1 and (I) GFP~TVCV MP localized to puncta (arrowheads) that were not observed in plants expressing free GFP (L). Hand-made cross sections (A-B,D-E,G-H,J-K) were imaged by using red and green channels (left column) and green channel only (middle column) of the confocal microscope. Paradermal images are shown in (C,F,I,L). Left panels of A and D show bright-field images of mGFP5-ER and GFP~KN1 plant sections, respectively.
(M-O) Immunolocalization using an anti-KN1 antiserum was performed on leaf sections of wild-type (M, red box is magnified in upper panel of O) and GFP~KN1 expressing plants (N, red box is magnified in lower panel of O). GFP~KN1 protein was detected in nuclei of epidermal cells and in mesophyll cells. Arrows indicate nuclear localization of GFP and GFP fusions (F, I, L) and KN1 protein (O). Scale bars: 50 μm (A,B,D,E,G,H,J,K,M); 25 μm (C,F,I,L,N,O).

the guard cells of the epidermis (Fig. 2A-C). In contrast, the *LTP1* and *AtML1* promoters drove epidermis-specific expression in unexpanded leaves, fully expanded leaves and hypocotyls (Fig. 3A-C); no green fluorescence above background was detected in sub-epidermal tissues. The *AtML1* promoter was stronger in the cotyledons and young leaves, while the *LTP1* promoter drove stronger expression in mature leaves.

Free GFP, GFP~KN1 and GFP~MP traffic from mesophyll to epidermal cells in the leaf

To determine whether GFP, GFP~KN1 or GFP~TVCV MP

could traffic from mesophyll to epidermal cells in the shoot, we imaged hand-cut cross sections from the respective UAS-transgene seedlings carrying the p*RbcS2b* GAL4 driver (Fig. 2). (The term 'driver' means a promoter in the GAL4/UAS two-component system.) We observed movement of GFP~KN1 (Fig. 2D-F), GFP~TVCV MP (Fig. 2G-I) and free GFP (Fig. 2J-L) to the epidermis. In each case, the level of fluorescence in the epidermal cells was approximately equal to that in the mesophyll. We assessed the sub-cellular localization of these fusion proteins at a higher magnification in paradermal confocal sections. Both GFP~KN1 and GFP~TVCV MP localized to the nuclei, the cytoplasm and to punctate spots
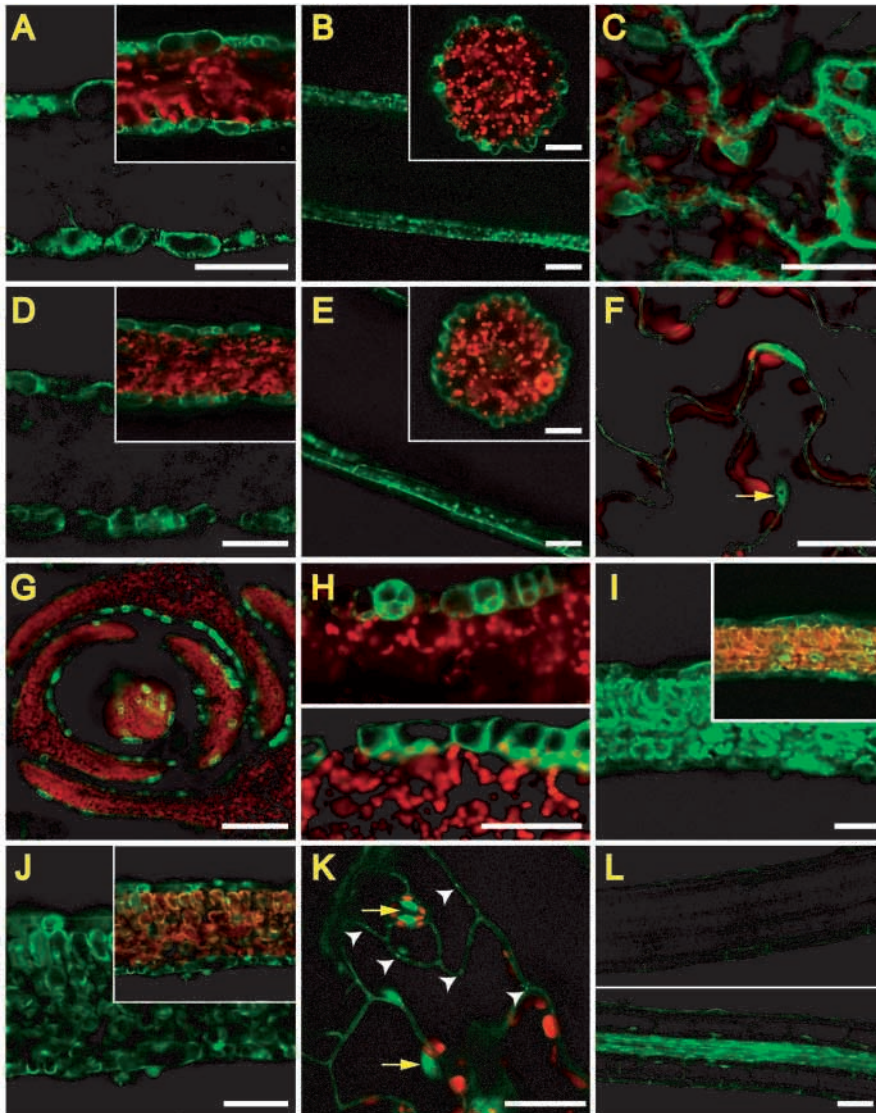
Fig. 3. GFP~KN1 does not traffic from epidermal to sub-epidermal cells in the leaf. GFP fusion reporters were expressed using the epidermis-specific p*LTP1* or p*AtML1* promoters in the GAL4/*UAS*-reporter system. Expression of the cell-autonomous mGFP5-ER reporter (A-C) under the control of p*LTP1* showed the epidermal specificity of pLTP1 in expanded leaf (A,C) and in hypocotyl (B). Similarly, GFP~KN1 was restricted to epidermal cells of expanded leaf (D) and hypocotyl (E). Note that the weak fluorescence in mesophyll cell layers in A and D is autofluorescence that is inevitable in sectioned plant tissues. (F) Epidermally expressed GFP~KN1 does not show punctate cell wall spots of green fluorescence. p*AtML1* expression similarly resulted in epidermis-specific localization of GFP~KN1 in young leaf primordia (G,H, lower panel) and mGFP5-ER (H, upper panel). (I-K) In contrast, GFP (I) and GFP~TVCV MP (J) expressed under the control of p*LTP1* showed extensive movement and GFP~TVCV MP localized to PDs (K). (L) A control root expressing mGFP5-ER under the control of *pAtML1* does not show any green fluorescence over background levels (L, upper panel). In contrast, the GFP~TVCV MP fusion produced in shoot epidermal cells could also be detected in root vascular and cortical tissues (lower panel), indicating its long distant trafficking. C,F,K are paradermal and A,B (inset) D, E (inset) and G-J are cross sections. Inset images and paradermal images are red/green channel images. Yellow arrows indicate nuclei; arrowheads show punctate cell wall spots. Scale bars: 25 μm (C,F,K,H); 50 μm (A,B,D,E,I,J,L); 100 μm (G).

between cells (Fig. 2F,I). Similar spots were not seen in plants expressing mGFP5-ER or free GFP (Fig. 2C,L). The GFP~KN1 fusion protein was also detected in epidermal cell nuclei using the anti-KN1 antibody (Fig. 2N,O, lower panel) (Smith et al., 1992), suggesting that the epidermal GFP fluorescence in these lines represented the intact GFP~KN1 fusion protein and not a free GFP degradation product. This observation of GFP~KN1 trafficking was consistent with results using an enhancer trap line to express this fusion in perivascular cells (Kim et al., 2002), and here we showed that both free GFP and GFP~TVCV MP were also able to move from mesophyll to epidermal cells.

GFP~KN1 did not traffic out of the epidermis of leaf primordia or of fully expanded leaves

GFP~KN1 could traffic in the leaf from the mesophyll to epidermal cell layer, where it accumulated in nuclei and in punctate cell wall spots. To investigate whether trafficking could also occur in the opposite direction, from epidermis to mesophyll, we expressed GFP~KN1 and the other fusion proteins in the epidermal layer of the shoot using the p*LTP1* and p*AtML1* GAL4 drivers. In leaves and hypocotyls, the p*LTP1* and p*AtML1* GAL4 drivers induced mGFP5-ER expression specifically in epidermal cells, with no green fluorescence over background levels detected in any other tissues of the shoot (Fig. 3A-C).

We next imaged fluorescence of GFP~KN1 under the control of the same L1-specific GAL4 drivers. GFP~KN1 fluorescence was restricted to the epidermal tissues of the mature leaf and hypocotyl, and we did not detect any movement to mesophyll cells (Fig. 3D-F). Therefore, in contrast to the result from mesophyll-specific expression, when expressed specifically in epidermal cells, GFP~KN1 was unable to traffic into cells in the adjacent cell layers.

To determine whether the absence of trafficking of GFP~KN1 out of epidermal cells also occurred in the early stages of leaf development, we imaged GFP fluorescence from young leaf primordia approximately 2 mm long. Expression of mGFP5-ER showed that p*AtML1* was also epidermis specific in young leaf primordia (Fig. 3H, upper panel). GFP~KN1

expressed under the control of p*AtML1* was also restricted to the epidermal layer (Fig. 3G,H, lower panel).

Plants expressing GFP~KN1 under the control of a mesophyll or perivascular tissue-specific GAL4 driver showed punctate localization in the cell wall and we investigated whether plants expressing GFP~KN1 in the epidermis also have this phenotype. We could not detect spots of GFP fluorescence in the cell walls of plants expressing GFP~KN1 in the epidermis (Fig. 3F), although epidermally expressed GFP~MP did show this putative plasmodesmal localization (Fig. 3K).

To test whether the restriction of protein trafficking out of epidermal cells was a general phenomenon, we expressed free GFP and GFP~TVCV MP using the L1-specific GAL4 drivers. Previous reports (Oparka et al., 1999) and our unpublished results suggested that free GFP could move freely from epidermal to mesophyll cells in bombardment assays, and we observed similar results in stable transgenic lines (Fig. 3I). We also detected extensive trafficking of GFP~TVCV MP from the epidermis into mesophyll and vascular tissues (Fig. 3J). In these cases, the green fluorescence intensity in mesophyll and vascular tissues was approximately equal to that in epidermal cells, suggesting that GFP and GFP~TVCV MP moved readily from epidermal to mesophyll cells. Therefore, the PDs between epidermal and mesophyll cells were open to both non-selective and selective movement.

Previous studies underline the importance that viral MPs play in long distance viral movement and infection (Deom et al., 1994; Wang et al., 1998; Itaya et al., 2002). Since we saw a high level of GFP fluorescence from the GFP~TVCV MP fusion in mesophyll and vascular tissues of the shoot, we investigated how far GFP~TVCV MP could traffic in the plant, by imaging roots of p*AtML1*-GFP~TVCV MP plants. *AtML1* mRNA is not detectable in the root (Lu et al., 1996), and mGFP5-ER expression was not detected in the mature region of p*AtML1*-mGFP5-ER roots (Fig. 3L, upper panel). However in seedlings carrying the *UAS*-GFP~TVCV MP construct, green fluorescence was detected in vascular and cortical tissue throughout the length of the root (Fig. 3L, lower panel), indicating that MP trafficking occurred over a long distance from the shoot epidermis into the root, presumably through the phloem. Since MP binds to MP RNA and facilitates its cell-to-cell trafficking (Nguyen et al., 1996), it is possible that RNA trafficking is responsible for some of the non autonomous effects.

In summary, whereas GFP~KN1 could traffic freely from mesophyll to epidermal cells, it could not traffic in the opposite direction. The epidermal/mesophyll interface was not blocked to either free diffusion-mediated or selective movement, since both free GFP and GFP~TVCV MP moved readily from epidermis to mesophyll and vascular tissues.

GFP fusions to ~KN1, ~STM and ~KNAT1 trafficked out of the epidermal (L1) layer in the shoot apical meristem

To investigate whether the restriction of trafficking of GFP~KN1 out of the epidermal layer occurred throughout all stages of shoot development, we imaged the SAM of p*AtML1*-GFP~KN1 plants. Our previous results indicated that GFP~KN1 could traffic into the L3 layers of the inflorescence meristem when expressed in L1 and L2 using the

SCARECROW promoter (Kim et al., 2002). However, that study did not determine specifically whether GFP~KN1 could traffic out of the L1. In the SAM, p*AtML1* induced expression of the mGFP5-ER reporter specifically in the L1 (Fig. 4A) showing strong perinuclear fluorescence (Fig. 4A, inset). A GUS~GFP fusion was similarly restricted to the L1 (Fig. 4B). In contrast to the situation in the leaf, we found that GFP~KN1 could traffic from the L1 into the L2 and L3 layers (Fig. 4C). We also tested if this cell-to-cell trafficking property of KN1 was conserved in its *Arabidopsis* homologs. GFP~KNAT1 and GFP~STM expressed using p*AtML1* did traffic in the SAM and showed strong L1 and weaker L2 fluorescence (Fig. 4D,E and inset). L3 GFP fluorescence from GFP~KNAT1 and GFP~STM was not evident from the confocal images, but GFP~KNAT1 was detected using a two-photon microscope (Fig. 4F). Quantification of the two-photon signal indicated that the photon number in the outer cell layer of the L3 was at least two-fold higher than background levels (Fig. 4G). The nuclear accumulation of the GFP-tagged protein was less evident in the two-photon image than in the confocal image because the two-photon microscope collects light from a narrower Z-section (1 μm). However, nuclear signal was evident in some cells in the two-photon image (arrowed in Fig. 4F). We also expressed GFP~MP and GFP in the L1 layer, and these proteins could also traffic out of the L1. The fluorescence patterns in apices expressing GFP or GFP~MP demonstrated more extensive movement than the GFP~KNOX fusions, through at least 6 cell layers (Fig. 4H,I). Two to three apices from each of 4 independent transgenic lines showed similar patterns of GFP or GFP~MP localization.

GFP~KN1 trafficking from the L1 into the L2/L3 occurred only in the SAM indicating that this aspect of KN1 trafficking was under developmental control. KNAT1 and STM could also traffic in the SAM, and trafficking of the KNOX proteins was more restricted than that of GFP or GFP~MP. The conservation in trafficking ability between functionally related KNOX proteins in different species suggests that trafficking may be associated with their innate functions.

L1-specific expression of KN1 and GFP~KN1 can partially complement *stm-11*

What are the potential functions of KN1 trafficking during development? As a first step to answer this question, we investigated whether KN1 continues to function in SAM maintenance after cell-to-cell trafficking. We first tested for complementation of *stm* mutants by KN1 or GFP~KN1. STM is expressed in the central and peripheral regions of the SAM, in L1, L2 and L3 layers (Long et al., 1996). However, the *STM* promoter used in this study drove strong GUS expression only in the peripheral region (Fig. 5A; A. Fernandez and M. K. Barton, personal communication).

As *stm-11* mutants are seedling lethal, we transformed *stm-11* heterozygotes with a p*STM*-GFP~KN1 construct. In the transgenic seedlings, the GFP~KN1 expression pattern was similar to that of p*STM*-GUS, except that fluorescence was also evident in the central region (Fig. 5B, arrow), suggesting that GFP~KN1 was trafficking from the peripheral to central region.

To determine whether *stm-11* mutants were complemented by KN1 or GFP~KN1, we genotyped the transgenic plants using a CAPS assay (A. Fernandez and M. K. Barton, personal
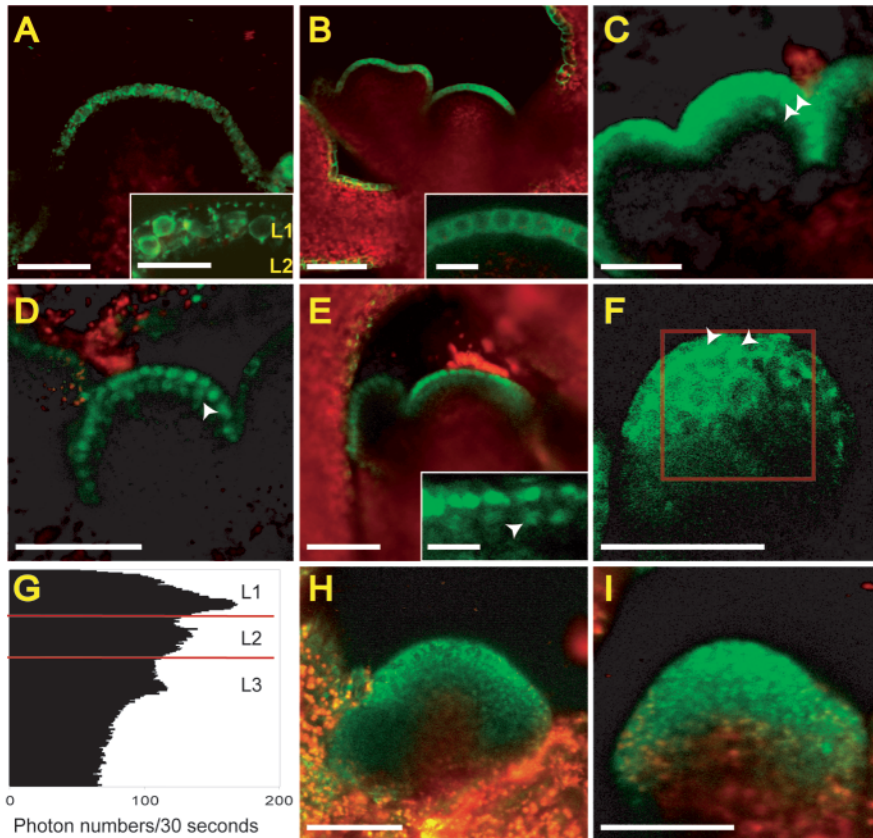
Fig. 4. GFP fusions to KNAT1, STM and KN1 are able to traffic in the inflorescence SAM. All reporter constructs were expressed under the control of p*AtML1*. (A,B) Cell-autonomous mGFP5-ER (A) and GUS~GFP (B) reporter expression demonstrates the L1 specificity of p*AtML1*. The high magnification views (A-B, insets) show predominant perinuclear or cytoplasmic green fluorescence of mGFP5-ER or GUS~GFP. (C-E) The GFP fusions to KN1 (C), STM (D) and KNAT1 (E) showed strong L1 and weaker L2 green fluorescence suggesting short-range movement. (F,G) GFP~KNAT1 is detected in L3 as well as L1 and L2 in the two-photon microscope image (F), and is quantified (G) in the region corresponding to the red-box in F. Nuclear localization of GFP fusions to KNOX proteins is also evident in the cross section images in L1 and L2 cells (C-F, arrowheads). L1 expression of GFP~TVCV MP (H) or GFP (I) resulted in more extensive movement in the SAM than the GFP~KNOX fusions. Scale bars: 25 μm (A), 50 μm (B-I), 10 μm (insets).

communication). We observed shoot rescue in *stm-11* homozygotes carrying the p*STM*-GFP~KN1 transgene, and a representative plant is shown in Fig. 5C. Similar rescue phenotypes were observed in 50% of the independent transgenic lines (4 lines total). The complemented plants showed relatively normal phyllotaxy, leaf and flower morphology (Fig. 5C, inset), but were shorter, had additional axillary shoots and were sterile. In contrast, transgenic lines carrying p*STM*-KN1 showed full complementation of *stm-11* (not shown), including normal stature, flower morphology and fertile seeds. Therefore KN1 could fully complement *stm* when expressed from the *STM* promoter, and the fusion of GFP to KN1 slightly impaired its function.

To address whether KN1 expression in the L1 and its subsequent trafficking into L2 and L3 layers was sufficient to rescue *stm*, we next transformed *stm-11* heterozygotes with the p*AtML1*-GFP~KN1 construct. In the T_1 generation 21/32 (66%) of the *stm-11* homozygotes showed partial complementation of the *stm-11* phenotype (Fig. 5D). All complemented plants showed abnormal phyllotaxy and lacked flowers or had abnormal, sterile flowers (Fig. 5D, right) that were similar to those of the weak *stm-2* mutant (Clark et al., 1996; Endrizzi et al., 1996). Therefore, the L1 expression of GFP~KN1 gave partial complementation of *stm-11* that was less complete than the complementation we observed using with p*STM*-GFP~KN1. To identify whether the partial complementation by L1 expression of GFP~KN1 was because of the GFP fusion to KN1, we also transformed *stm-11* heterozygotes with a p*AtML1*-KN1 construct. The T_1 *stm-11*

seedlings (>20 independent lines) carrying the p*AtML1*-KN1 construct showed similar phenotypes to those with p*AtML1*-GFP~KN1, i.e. they also showed only partial complementation (Fig. 5D, left). We also analyzed the T_2 generation from the *stm-11* heterozygous T_1 plants carrying p*AtML1*-KN1. About 32% (74/229) of *stm* homozygous seedlings from 3 independent lines showed partial complementation, with phenotypes similar to those observed in the T_1 (not shown).

Since STM and KNAT1 could also traffic out of the L1, we determined whether L1 expression of these proteins could complement *stm-11*. Plants expressing STM or GFP~STM from p*AtML1* showed a very severe phenotype of stunted growth, small lobed leaves, no cauline leaves and flowers defective in sepal, petal and stamen development (Fig. 5E). This phenotypes was presumably partly due to ectopic expression of STM in the leaves and floral organs. A few plants showed milder, bushy phenotypes (Fig. 5F). These bushy plants were homozygous *stm-11* (not shown), suggesting therefore partial complementation by the L1-specific expression of STM. L1 expression of KNAT1 also resulted in partial complementation, as about 30% (7 /24) of T1 plants expressing GFP~KNAT1 in the L1 showed partial rescue of *stm-11*. The *stm-11*/ p*AtML1*-GFP~KNAT1 seedlings developed rosette shoots (Fig. 5G) that later became bushy, similar to *stm-2* plants (not shown).

In summary, L1 expression of KN1, STM or KNAT1 was sufficient to give partial rescue of *stm-11*. We assume that this rescue is in part due to KNOX protein that traffics into the L2 and L3 layers of the SAM. The partial complementation of *stm-*
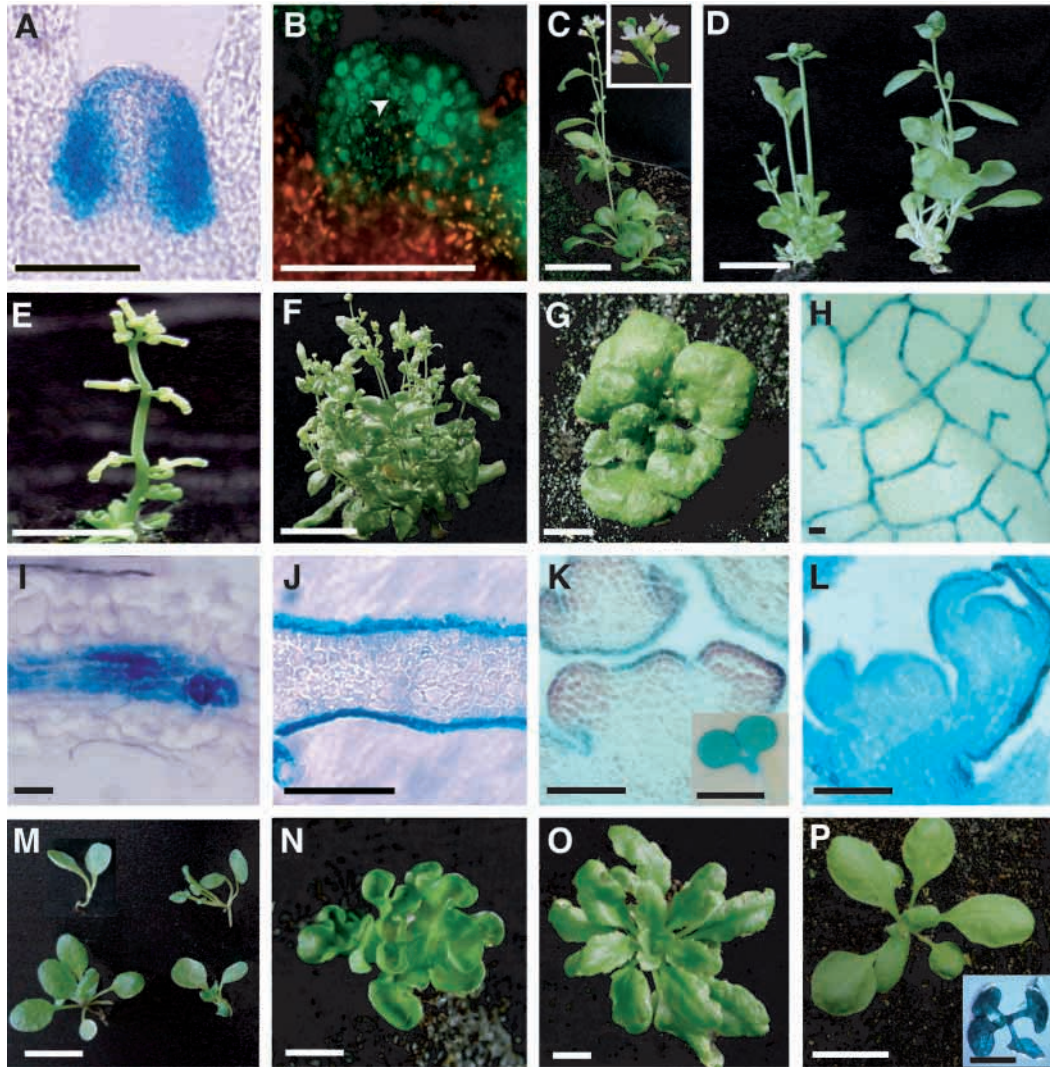
Fig. 5. L1-specific expression of KN1, STM or KNAT1 is sufficient to partially complement *stm-11*. (A) GUS expression from the *STM* promoter (p*STM*) was detected mainly in the peripheral region of the SAM. (B) p*STM* driven GFP~KN1 expression shows a similar pattern to that of GUS, but with GFP fluorescence also in the central region (arrowhead). (C) The p*STM*-GFP~KN1 transgene complemented *stm-11*, producing *stm* homozygous plants with shoots showing relatively normal phyllotaxy and flower morphology (inset) (an *stm-11* seedling with fused cotyledons and no true leaves is shown in M, upper left). (D) L1-specific expression of KN1 (left) and GFP~KN1 (right) under the control of p*AtML1* partially rescued *stm-11*. (E) p*AtML1*-GFP~STM (or STM) expression resulted in a severe phenotype in most plants, although some of the partially rescued *stm-11* homozygous plants had a bushy phenotype (F). (G) L1-specific GFP~KNAT1 expression could also partially rescue *stm-11*. (H-K) The GUS~KN1 fusion protein was cell-autonomous in the leaf (H-J) and in the SAM (K). Perivascular-specific GUS staining of the leaf of a J2111/*UAS*-GUS~KN1 plant seen from the top down (H) and in cross section view (I). (J,K) In p*AtML1*-GUS-KN1 plants, GUS activity was epidermis specific in the leaf (J) and in the shoot apex (K). The inset in K shows epidermal GUS~KN1 expression in a whole-mount *stm-11* seedling. (L,M) In *35S*-GUS~KN1 plants, GUS activity was detected in all SAM layers (L), and *35S*-GUS~KN1 could partially rescue *stm-11*; two rescued *stm-11* seedlings (right), *stm-11* seedling (top left) and wild-type seedling (bottom left) are shown in M. However, over-expression of GUS~KN1 in the leaf does not lead to over-expression phenotypes. (N,O) A J2111/*UAS*-GFP~KN1 plant shows KN1 over-expression phenotypes (N), while J2111/*UAS*-GUS~KN1 plants had wild-type morphology (O). (P) Similarly, *35S*-GUS~KN1 overexpressing plants (*stm-11/STM* genotype) did not show any KN1 over-expression phenotypes, despite showing high GUS activity (inset). Scale bars: 50 µm (A,B,H-L), 0.5 cm (F and insets in K,P), 1 cm (E,G,M-P), 3 cm (C,D).

11 by KNAT1 expression agrees with previous findings that KNAT1 has a partially redundant function with STM (Byrne et al., 2002).

To test if KN1 trafficking was essential for complementation of *stm-11* in this assay, we made a non-trafficking GUS~KN1 fusion. We fused GUS (68 kDa) at the N terminus of KN1, with an intervening alanine linker, as previous studies showed this to be the optimal configuration for KN1 fusions (Kim et al., 2002). The GUS~KN1 fusion protein behaved cell-autonomously in the leaf, as GUS activity was detected only in the perivascular cells in J2111/ *UAS*-GUS~KN1 lines (Fig. 5H,I) or in the epidermis in p*AtML1*-GUS~KN1 lines (Fig. 5J). The GUS~KN1 fusion was also cell-autonomous in the SAM, as p*AtML1*-GUS~KN1 plants showed GUS activity only in the L1 layer (Fig. 5K).

We next asked if the L1 restricted expression of GUS~KN1 could rescue *stm-11*. We observed no rescue of the *stm-11* phenotype in 16 independent T₁ *stm-11* seedlings and in more than 200 *stm-11* seedlings from 4 independent T₂ lines that expressed GUS~KN1 strongly in the L1 (Fig. 5K, inset). This failure to complement could be because GUS~KN1 was unable to traffic into L2 and L3 layers, or because the GUS fusion blocked KN1 function independently of its inhibition of KN1 trafficking. To distinguish these possibilities, we asked if expression of GUS~KN1 in all cell layers of the SAM was sufficient to rescue *stm-11*. To this end, a *35S-GUS~KN1* construct was transformed into *stm-11* heterozygotes. As expected, the transgenic plants showed constitutive GUS activity in all SAM layers (Fig. 5L). We confirmed GUS~KN1 over-expression by western blotting using the anti-KN1 antibody (Smith et al., 1992) (data not shown). We observed shoot rescue in five out of 15 independent T2 lines (33%) that segregated for *stm-11*, indicating that the GUS~KN1 fusion was indeed functional in this shoot rescue assay (Fig. 5M). The rescue phenotypes were generally weaker than those of the GFP~KN1 lines, though some of the 35S-GUS~KN1-rescued seedlings developed inflorescence shoots, similar to the *stm-11* plants rescued by L1-specific expression of KNOX proteins (not shown). Therefore, GUS~KN1 expression in all SAM layers could partially complement *stm-11*, whereas L1-specific expression gave no such complementation.

We previously showed that *35S*-GFP~KN1 plants develop KNOX over-expression phenotypes (Kim et al., 2002). To our surprise, the *35S*-GUS~KN1 transgenic plants (>40) that were wild type for *stm* did not show any KN1 over-expression phenotypes (Fig. 5P), despite having strong expression of GUS~KN1 throughout the leaves (Fig. 5P, inset).

In summary, GUS~KN1 was cell-autonomous and partially complemented *stm-11* if expressed in all meristem layers, but not when expressed specifically in the L1. These data support the hypothesis that trafficking out of the L1 is required for shoot rescue in the p*AtML1*-KNOX expression lines. Over-expression of the GUS~KN1 fusion did not produce KNOX over-expression phenotypes, suggesting either that the over-expression phenotypes require the trafficking function of KN1, or that the GUS fusion interrupts some other function of KN1 involved specifically in generating over-expression phenotypes.

DISCUSSION

The intercellular trafficking of regulatory proteins likely transmits important developmental signals in plants. Protein trafficking is thought to occur through plasmodesmata, and if developmentally significant, should be under strict temporal and/or tissue-specific control. Developmental modifications in PDs do indeed affect free GFP diffusion and selective MP trafficking (Itaya et al., 1998; Oparka et al., 1999), however almost nothing is known about whether and how regulation occurs for plant proteins. Here we showed that KN1 trafficking is not only under developmental control but also that KNOX protein trafficking regulates the fate of target cells in the meristem, indicating that the trafficking of homeodomain proteins has potential developmental significance.

Developmental and tissue-specific control of KN1 trafficking

In plants with simple leaves, KNOX genes are usually expressed only in the SAM, though developmental consequences of ectopic leaf expression and the expression of KNOX genes in compound leaves indicate that they can function during leaf development (Bharathan et al., 2002). We found that GFP~KN1 trafficking was regulated tissue-specifically in the leaf. Whereas it could traffic from mesophyll to epidermis, trafficking did not occur from epidermis to mesophyll, in either leaf primordia or in fully expanded leaves. Consistent with these observations, distinctive phenotypes arose from layer-specific expression of KN1. As expected, the mesophyll-specific expression of GFP~KN1 resulted in strong KNOX over-expression phenotypes, similar to constitutive (35S promoter driven) expression (Lincoln et al., 1994; Chuck et al., 1996). However, epidermal-specific expression of GFP~KN1 produced a relatively mild, rumpled phenotype. This mild phenotype is probably due to the restriction of KN1 in the epidermis, though the fact that epidermal expression is able to alter leaf shape does indicate a non autonomous effect of KN1 that is presumably not due to trafficking. Similar uni-directional signaling was observed in periclinal chimeras of the floral organ identity genes *GLOBOSA* (*GLO*) and *DEFICIENS* (*DEF*) in *Antirrhinum*. Some effects of DEF and GLO expression were non cell-autonomous, however these effects were only partially explained by movement, as DEF also moves from L2 to L1 but not in the opposite direction (Perbal et al., 1996). In this study we showed that the lack of protein movement out of the epidermis was not, however, a general phenomenon. Both GFP and GFP~TVCV MP moved freely from epidermis to mesophyll, demonstrating that the PDs between epidermal and mesophyll cells are open to both diffusion-mediated and selective protein movement.

Uni-directional trafficking of KN1 in the leaf suggests that KN1 normally traffics from inner to outer layers, and may reveal a directional signaling pathway. Although class I KNOX genes are not normally expressed in the simple leaves of maize, rice and *Arabidopsis*, they are expressed in compound leaves (Bharathan et al., 2002). For example, LeT6 is expressed in tomato leaf primordia as well as in the SAM (Chen et al., 1997; Kim et al., 2001). Chen et al. showed that *LeT6* mRNA expression was strong in the L2/L3 layers and reduced or absent from the L1 layer of the SAM, and we note that this expression pattern continues in the young leaf primordia (Chen et al., 1997). It would be interesting therefore to test whether KNOX proteins traffic directionally in the tomato compound leaf; such a process could provide a mechanism for regulation of leaf morphology by signaling from inner to outer layers during development. Classic studies involving inter-specific periclinal leaf chimeras indeed indicate the potential for direction signaling during leaf morphogenesis (reviewed by Tilney-Bassett, 1986).

In support of the hypothesis that KN1 traffics through PDs, it showed a punctate pattern resembling that seen with GFP~MP, which has been linked with plasmodesmata in studies using electron microscope level immunolocalization (Ding et al., 1992a). Our results imply, however, that the mechanism of KN1 trafficking differs from that of the viral MP. This could be because tissue-specific receptors that recognize distinct trafficking motifs in the different proteins. This idea is

supported by the recent report that non-cell-autonomous pathway protein (NCAPP1), a putative PD receptor, interacts with CmPP16 and TMV MP but not with KN1 (Lee et al., 2003). Alternatively, tissue-specific post-translational modification(s) of KN1 might affect its ability to traffic. For example, phosphorylation regulates the trafficking of TMV MP (Citovsky et al., 1993; Waigmann et al., 2000), and perhaps a similar mechanism controls KN1 trafficking.

KN1 and related *Arabidopsis* KNOX proteins STM and KNAT1 traffic in the SAM

Whereas KN1 was unable to traffic from epidermal to mesophyll cells in the leaf, it could traffic from epidermal (L1) cells to underlying cells in the inflorescence SAM. Two *Arabidopsis* homologs of KN1, KNAT1 and STM, could also traffic in the SAM, suggesting that hypothetical signal(s) for trafficking in KN1 are conserved in other KNOX proteins. These signal(s) could be made up of a simple, short sequence and/or complex structural motif(s). So far, studies using viral MPs and rice phloem proteins suggest that recognition by the PD trafficking machinery involves structural motifs (Haywood et al., 2002; Ishiwatari et al., 1998), though a short sequence motif appears to control the trafficking of the heat shock cognate 70 chaperone (Aoki et al., 2002). In the case of KN1, a short peptide, homologous to a region near the N terminus of the protein, can interfere with its trafficking, though it is not known whether this sequence motif is sufficient for trafficking (Kragler et al., 1998).

We observed a relatively short range of GFP~KNOX protein trafficking in the *Arabidopsis* SAM, which generated a steep gradient of GFP fluorescence spanning approximately 2~3 cell layers. In contrast, GFP~KN1 can traffic over at least 3-5 cell layers in the leaf (Kim et al., 2002). GFP~TVCV MP or free GFP moved further than GFP~KNOX in the SAM, through more than six cell layers. This suggests that KNOX protein trafficking in the meristem is relatively restricted, and may be used for short range signaling. The more pronounced nuclear localization of GFP~KN1 in the meristem than in the leaf may be the cause of its shorter range of trafficking, as nuclear localization probably restricts its chance to interact with PD. A similar mechanism was proposed for the restriction of SHR trafficking (Nakajima et al., 2001) and of GFP diffusion (Crawford and Zambryski, 2000). In the maize shoot apex, a KN1 protein gradient is also evident between the SAM and leaf primordia (Jackson, 2002). Although the biological significance of these KNOX protein gradients is not yet clear, it is possible that they are used to activate target genes at different positions along the gradient (Jackson, 2002).

Caution is required in interpreting the trafficking of GFP fusion proteins. The GFP tag increases the size of the protein and may affect trafficking efficiency. However, the GFP~KN1 fusion used in this study produced normal KN1 overexpression phenotypes and also complemented the *stm* mutation, suggesting it retained normal biological function(s). In addition, the low quantum yield for GFP, relative to other fluorophores, may well result in a significant underestimation of the range over which the tagged protein can traffic.

Biological function of KNOX homeodomain protein trafficking

The critical question is what, if any, is the function of KNOX

protein trafficking in intercellular signaling? The conservation of trafficking ability in KNOX proteins of different species suggests that their function requires trafficking, and the nuclear localization of GFP~KNOX proteins following trafficking in the SAM suggests that they can function in transcription in target cells. Cell-to-cell trafficking of KNAT1 could explain the non cell-autonomous regulation of epidermal cell fate by KNAT1, reported by Venglat et al. (Venglat et al., 2002).

GFP~KNOX expression in the L1 rescued shoot formation in *stm-11* mutants, and trafficking was probably required for this rescue, because the cell-autonomous GUS~KN1 fusion did not result in rescue. Whereas this failure to rescue could be because GUS~KN1 was inactive, this was not the case since it could partially rescue *stm-11* when expressed in all meristem cell layers using the *35S* promoter. However, the rescue of *stm-11* by *35S*-GUS~KN1 was only partial, and we speculate that full KN1 function in the SAM might require its intercellular trafficking. A functional requirement for KN1 trafficking in the wild-type situation remains to be tested by determining if expression of the non-trafficking version of KN1 under a faithful *STM* promoter can complement the *stm* mutant.

Our results suggest that the correct spatial and temporal expression pattern and/or level of KN1/STM in the SAM is required for full complementation of *stm-11*. In contrast to the *AtML1* promoter, use of the *STM* promoter resulted in GFP~KN1 expression at a relatively homogeneous level in the different SAM layers. These expression differences are probably the reason why the two constructs resulted in different complementation phenotypes; p*AtML1*-GFP~KN1 (or KN1) expression always resulted in partial complementation of *stm* and abnormal phyllotaxy, while complementation using p*STM*-GFP~KN1 (or KN1) was more complete and produced seedlings with normal phyllotaxy. In this regard, intercellular protein trafficking might provide a way to regulate the distribution and concentration of key developmental regulators across a cellular domain like the SAM. Intercellular KN1/STM trafficking may be a redundant 'fail-safe' mechanism to ensure all cells adopt the SAM fate, analogous to the mechanism originally proposed by Mezitt and Lucas (Mezitt and Lucas, 1996) for non-autonomous action of FLORICAULA, and supported by the demonstration of LFY trafficking (Sessions et al., 2000).

Plants expressing the non-trafficking *35S*-GUS~KN1 fusion did not have the usual KN1 over-expression phenotypes. We therefore hypothesize that KN1 trafficking might be required to generate the over-expression phenotypes, or that movement per-se is important for function, rather than simply which cells contain KN1. One possible mechanism would be if KN1 was modified and gained a novel function during intercellular movement. Partial unfolding of KN1 is required for passage through PDs (Kragler et al., 1998), and could expose KN1 to post-translational modification(s). Alternatively, the lack of a KNOX overexpression phenotype in the *35S*-GUS~KN1 plants could be because the fusion of GUS inhibits the ability KN1 to interact with partner proteins, such as *Arabidopsis* homologs of KN1 interacting protein (KIP), a BEL1-like TALE homeodomain protein (Smith et al., 2002). Such interacting proteins may be differentially expressed or differentially required for the over-expression and SAM functions of KN1. Lastly, the nuclear localization of STM is required for its activity (Gallois et al., 2002), and if the fusion of GUS

interfered with KN1 nuclear localization specifically in the leaf this could also affect its ability to generate over-expression phenotypes.

In conclusion, KN1 trafficking is under temporal and tissue-specific developmental control, and trafficking ability is conserved in STM and KNAT1. Our results suggest that trafficking of KNOX homeodomain proteins is functionally significant, and may coordinate the development of source and target cells, or provide a redundant 'fail-safe' mechanism to control the fate of cells in the SAM.

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