Double Labeling of KNOTTED1 mRNA and Protein Reveals Multiple Potential Sites of Protein Trafficking in the Shoot Apex

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Recent reports indicate that several plant mRNAs and proteins are able to traffic intercellularly through plasmodesmata. Localization studies can reveal differences between mRNA and protein localization that would be indicative of such a process. However, subtle differences could be missed when comparing localization in adjacent sections, especially in developmental studies where adjacent sections through immature apical regions may be one or more cells removed from each other. Therefore, we have developed a novel method for double localization of KNOTTED1 mRNA and protein in sections through the maize (Zea mays) shoot apex. The advantage of double labeling is revealed in our demonstration of novel potential sites of cell-to-cell trafficking of KNOTTED1 protein in the shoot apical region. The technique should be applicable to any gene products where the appropriate probes are available and will, therefore, help to determine the extent of protein and/or mRNA trafficking in plants.

Cell-to-cell communication is essential to coordinate the development and physiology of multicellular organisms. A recently highlighted pathway for communication in plants is through plasmodesmata (PDs), intercellular channels that connect the majority of plant cells to their neighbors. PDs consist of a sleeve of plasmamembrane with a thin tube of apressed endoplasmic reticulum (ER) running through the center. Free movement of small molecules and regulated transport of macromolecules is thought to occur through the cytoplasmic space between the ER and plasmamembrane (Ding et al., 1999; Lucas, 1999; Jackson, 2000; Zambryski and Crawford, 2000).

Although PDs are likely to be essential coordinators of plant development and metabolism, they have been best characterized through the involuntary aid they afford to viruses, which view PDs as the gateway to a new host cell (Ghoshroy and Citovsky, 1997; Reichel et al., 1999). Many viruses encode movement proteins (MPs) that localize to PDs and move themselves and MP-viral nucleic acid complexes between cells. It is logically thought that MPs hitch a ride on an endogenous pathway for intercellular trafficking of macromolecules, and this is supported by the discovery of a phloem expressed gene, PP16, which shows both sequence and functional similarity to MPs (Xoxonostle-Cazares et al., 1999).

Growing evidence indicates a specific role for PDs in regulating symplasmic communication during development. Dye injection and loading studies have revealed symplasmic domains in the developing root and shoot that are dynamically controlled (Duckett et al., 1994; Rinne and van der Schoot, 1998; Gisel et al., 1999). How these studies relate to macromolecular trafficking is not known. What is clear, however, is that many developmental genes are involved in cell-to-cell communication, because they act nonautonomously. This includes several members of the KNOX class of homeodomain transcription factors that were first characterized by dominant mutations affecting cell fate in the maize (Zea mays) leaf (Sinha, 1999). In the case of Knotted1 (Kn1), we suggested a mechanism for its nonautonomy when we showed that KNOTTED1 (KN1) protein is detected outside of the domain of mRNA expression in the leaf (Jackson et al., 1994). In addition, KN1 protein injected into tobacco (Nicotiana tabacum) or maize mesophyll cells has the ability to traffic between cells, to gate PD, and to traffic its mRNA (Lucas et al., 1995). Thus, the cell to cell trafficking of KN1 suggests a molecular mechanism to explain its nonautonomy during leaf development.

KNOX genes have been described in many plants, including Arabidopsis, where there are at least three members of the class I group, SHOOTMERISTEMLESS (STM), KNAT1, and KNAT2 (Lincoln et al., 1994; Long et al., 1996). Class I KNOX genes are expressed in the shoot apical meristem (SAM), and the expression pattern and loss of function phenotypes of STM are most similar to those of kn1. In both cases, strong alleles fail to initiate the SAM, whereas weaker alleles have phenotypes that suggest an additional role in SAM maintenance (Clark et al., 1996; Endrizzi et al., 1996; Long et al., 1996; Kerstetter et al., 1997; Vollbrecht et al., 2000). An open question is whether KNOX gene products traffic in the SAM, where they normally function. Localization studies revealed symplasmic domains in the developing root and shoot that are dynamically controlled (Duckett et al., 1994; Rinne and van der Schoot, 1998; Gisel et al., 1999). How these studies relate to macromolecular trafficking is not known. What is clear, however, is that many developmental genes are involved in cell-to-cell communication, because they act nonautonomously. This includes several members of the KNOX class of homeodomain transcription factors that were first characterized by dominant mutations affecting cell fate in the maize (Zea mays) leaf (Sinha, 1999). In the case of Knotted1 (Kn1), we suggested a mechanism for its nonautonomy when we showed that KNOTTED1 (KN1) protein is detected outside of the domain of mRNA expression in the leaf (Jackson et al., 1994). In addition, KN1 protein injected into tobacco (Nicotiana tabacum) or maize mesophyll cells has the ability to traffic between cells, to gate PD, and to traffic its mRNA (Lucas et al., 1995). Thus, the cell to cell trafficking of KN1 suggests a molecular mechanism to explain its nonautonomy during leaf development.

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suggest this is the case for KN1 protein, because it is detected in the L1 layer of the SAM, where KN1 mRNA was not detected (Jackson et al., 1994; Lucas et al., 1995). In addition, a green fluorescent protein (GFP) fusion of KN1 was able to traffic between cells in the Arabidopsis shoot meristem (Kim et al., 2002).

GFP expression has provided novel insights into the regulation of PD size exclusion limit (SEL) during development. Estimates of SEL based on dye microinjection studies vary from one to a few kilodaltons in most cell types (Terry and Robards, 1987; Wolf et al., 1989; Waigmann and Zambryski, 1995). However, in plants where the phloem companion cell-specific SUC 2 promoter drives GFP expression, the GFP fluorescence spreads through the phloem into sink leaves and unloads into mesophyll and epidermal cells, suggesting that the SEL in those tissues is much higher (Imlau et al., 1999; Oparka et al., 1999). Using different GFP fusions, the SEL in leaves has now been estimated at 30 to 50 kD (Oparka et al., 1999; Itaya et al., 2000; Crawford and Zambryski, 2001). Moreover, the free movement of GFP is not always restricted to sink tissues, because it can also move freely between epidermal cells of Arabidopsis source leaves (Itaya et al., 2000). GFP movement indicates that the SEL is higher than once thought, however, the compact structure of GFP makes it difficult to relate these findings to other proteins. Other factors, such as subcellular localization, are also important in determining whether a protein will traffic (Crawford and Zambryksi, 2000). However, given that many plant proteins, including transcription factors, are below these new estimates of PD SEL, it is important to reassess the range and extent of cell-to-cell movement of regulatory macromolecules in vivo.

In summary, PDs are subject to dynamic regulation during development, and their SEL may be much higher than previously thought. To provide a more accurate view of potential sites of cell-to-cell movement of KN1 in the SAM, we developed a new double-labeling technique that allows the detection of mRNA and protein in the same tissue section. The use of these methods for localization of KN1 mRNA and protein suggest novel sites of cell-to-cell trafficking of KN1 protein in the shoot apex in addition to those already described.

RESULTS

To develop a double-labeling procedure, we initially reasoned that it would be better to perform the in situ mRNA detection before immunolocalization, because the mRNA in the tissue sections might be sensitive to degradation during immunological detection steps. However, we were unable to detect KN1 protein by immunolocalization after the in situ detection of KN1 mRNA, perhaps because the KN1 epitopes may have been destroyed during the relatively harsh in situ hybridization incubations. We, therefore, decided to perform the immunolocalization first, using heparin as an RNase inhibitor in the antiserum incubation. The basic procedure is outlined in Figure 1. To allow different color detection of the protein and mRNA, we used the peroxidase enzyme and diaminobenzidine (DAB) substrate, which generate a highly insoluble brown precipitate, for immunolocalization, followed by the traditional alkaline phosphatase, 4-nitroblue tetrazolium chloride, 5-bromo-4-chloro-3-indolyl-phosphate detection of KN1 mRNA, generating a blue signal (Jackson et al., 1994). The detection procedures were adapted from techniques developed for animal tissue sections and fruitfly (Drosophila melanogaster) embryo whole mounts (Brahic and Ozden, 1992; Manoukian and Krause, 1992).

Outline of the double labeling procedure.

1. Fix, dehydrate and embed tissue in wax, section and mount on coated slides.

2. Process for immunolocalization, including pre-digestion with proteinase K, and using heparin as an RNase inhibitor; detection of protein using peroxidase/ DAB substrate (brown product).

3. Block using hybridization solution then conventional in situ hybridization; followed by detection using alkaline phosphatase/ BCIP,NBT substrate (blue product).

4. Dehydrate, mount and photograph.

Figure 1. Outline of the double-labeling procedure.
To be sure that KN1 mRNA could not be detected in the L1 layer, we overexposed a section labeled in situ for KN1 mRNA by incubating the section for 5 d in substrate. This led to a very intense signal in the inner cells of the meristem, but no labeling was present in the L1 layer (Fig. 2A). Next, we performed the double-labeling protocol, and we found that the signal strengths for KN1 protein and mRNA detection were similar using these methods to those obtained with single labeling (Smith et al., 1992; Jackson et al., 1994). In agreement with previous observations from single labeling, KN1 protein but not mRNA was clearly detected in the L1 layer of the SAM (Fig. 2, B and C). However, in contrast to previous studies, the double labeling allowed greater resolution of the differences between protein and mRNA localization. In particular, clear differences were evident in the disc of insertion of the incipient leaf primordium, also known as P0. Here, the differences were distinct on either side of the disc of insertion. On the side that will give rise to the leaf margins (this side is adjacent to the central or mid-vein part of the P1 primordium and is on the left in Fig. 2B), there were two to three layers of cells that contained nuclear KN1 protein, but no detectable KN1 mRNA (Fig. 2, B and D). On the opposite side, the incipient midrib region of the P0, an area of two to three cells wide contained neither KN1 protein nor mRNA. Internal to this region was a strip of three to four cells wide that contained no detectable KN1 mRNA, but displayed a gradient of KN1 protein concentration (Fig. 2, B and E). At the base of older leaf primordia on the abaxial side, there was also a gradient of KN1 protein (Fig. 2, B and F). These gradients spread over about six cells outside of the domain of KN1 mRNA detection and were present at the base of P1, P2 (Fig. 2B), and older leaf primordia (not shown).

In summary, differences between KN1 mRNA and protein detection were observed in the L1 layer, at the disc of insertion of the P0 leaf, and at the base of developing leaf primordia. In all cases, the differences consisted of cells that labeled for KN1 protein but not mRNA; we never saw the converse situation. Gradients of KN1 protein concentration were observed at the boundaries between leaf primordia and the meristem or stem. The localization signals in this double-labeling procedure were similar to the respective single-labeling technique, therefore, these methods should be applicable to any gene products that can be detected by traditional localization techniques.

Figure 2. Double labeling of KN1 protein and mRNA in the maize shoot apex. A, A median section through a vegetative shoot apex, labeled for KN1 mRNA (blue). This in situ was overexposed to confirm the lack of KN1 mRNA in the L1 layer. Note that this apex was from a tetraploid seedling and that the meristem and primordia are about one-third larger than normal. B, An apex section double-labeled for KN1 protein (brown) and mRNA (blue). Note that KN1 protein is predominantly nuclear, whereas the mRNA is detected in the cytoplasm and nucleoplasm, as expected. An outline of this apex is displayed to the right of B, with the plastochron (P) 3, P2, P1 leaf primordia labeled and the incipient P0 disc of insertion shaded in gray. The regions that are enlarged in C through F are shaded pink. C, The presence of KN1 protein but not mRNA in cells in the L1 layer of the meristem (red arrowheads). Note that the L1 cells contain only brown staining in the nuclei, whereas cells in the L2 have in addition blue staining in the cytoplasm. D, The leaf margin side of the disc of insertion. Note that two to three layers of cells contain KN1 protein but no detectable mRNA; the mRNA expression domain is to the right of the red arrowheads. E, The mid-vein side of the P0. Note that there is a gradient of KN1 protein concentration spread over three to four cells (denoted by red-pink arrowheads). F, A gradient of KN1 protein is also evident at the base of leaf primordia, on the abaxial side; for example, at the base of the P2, the gradient is denoted by arrowheads of graded intensity. No KN1 mRNA is detected in these cells. Scale bar in A = 125 μm, in B = 100 μm, and in C through F = 25 μm.
DISCUSSION

We present a sensitive method for double labeling of protein and mRNA on the same tissue section, which should be applicable to any plant gene products for which suitable probes are available. This method is especially suited to looking for differences in localization that might be indicative of cell-to-cell trafficking of proteins or mRNAs. The methods include a traditional immunolocalization procedure, followed by an abbreviated in situ hybridization procedure where, in place of the usual permeabilization and blocking steps, a simple prehybridization incubation is used. In general, a protease digestion is required in in situ hybridization pretreatments to permeabilize the section for probe access; we omitted this step because our immunolocalization procedure already incorporated a protease predigestion. However, in cases where the immunolocalization procedure does not include a protease permeabilization, it should be possible to perform this step after the immunolocalization detection steps.

Using these techniques to detect KN1 mRNA and protein in the maize shoot apex, we confirmed that KN1 protein but not the corresponding mRNA was detected in the L1 layer of the SAM (Jackson et al., 1994; Lucas et al., 1995). We extended these findings by showing that KN1 protein was found outside of the domain of the mRNA expression in other regions of the shoot apex, in particular in the incipient leaf primordium or P0 and at the base of immature leaf primordia. The P0 forms a croissant-shaped ring of cells that encircles the periphery of the SAM and is initiating the new leaf primordium. *kn1* is a marker for the P0 because its expression is down-regulated in that region, consistent with its role in specifying the indeterminate fate of meristem cells (Smith et al., 1992; Sinha et al., 1993; Vollbrecht et al., 2000).

In the P0 domain, down-regulation of KN1 transcription and/or the degradation of KN1 mRNA were presumably responsible for the lack of KN1 mRNA in some P0 cells. Two possible mechanisms could explain the presence of KN1 protein in the P0 cells that lack KN1 mRNA. First, KN1 protein may simply be more stable than KN1 mRNA and, therefore, persists after the mRNA has been degraded. A second possibility is that KN1 protein traffics into cells in the P0 domain from adjacent SAM cells that accumulate high levels of KN1 mRNA and protein. Although we cannot distinguish between these two possibilities from these studies, our demonstration of cell-to-cell trafficking of KN1 in the leaf (Lucas et al., 1995) and in the SAM (Kim et al., 2002) makes it likely that protein trafficking makes some contribution to the observed distribution of KN1 protein. Further support for the hypothesis that KN1 trafficking has come from phage display experiments that identified sequence motifs in KN1 that interact with a putative PD receptor (Kragler et al., 2000).

KN1 protein and mRNA localization differs significantly on the margin compared with the mid-vein side of the P0. It is thought that leaf initiation starts in the mid-vein position and the disc of insertion of the P0 spreads around the SAM to later form the marginal domains (Jackson et al., 1994; Scanlon et al., 1996). Therefore, cells in the mid-vein region probably down-regulate *KN1* earlier than those in the margin region of the P0. In accordance, at any time point, the cells on the mid-vein side of the P0 have been without *kn1* expression longer, which probably explains why these cells have a lower level of KN1 products than the cells on the margin side. An intriguing observation is the presence of a gradient of KN1 protein concentration between the SAM and the P0 domain, which is evident on the mid-vein side in the example shown. Although this gradient could be attributable to a progressive inward spread of KN1 protein degradation, the fact that a KN1 gradient persists at the base of older leaf primordia and, therefore, over a period of many days argues against this possibility. Instead, the gradient may form as a result of KN1 protein trafficking from those cells nearby that contain a high level of KN1 mRNA and protein. If this is true, it suggests that the range of KN1 protein trafficking in the SAM was about three to six cells, the observed width of the KN1 gradient. Whether this KN1 protein gradient has a biological function is unknown, however gradients of homeodomain proteins are commonly used in developmental patterning, for example in the fruitfly syncytial embryo (Struhl et al., 1989). These gradients are used specifically to activate different target genes at different protein concentrations or positions along the gradient. Therefore, one possibility is that the KN1 gradient could provide positional information to activate boundary specific target genes. Candidate boundary genes (in Arabidopsis) include *UNUSUAL FLORAL ORGANS* and *CUP SHAPED COTYLEDON* (Lee et al., 1997; Aida et al., 1999).

Gradients of KN1 protein were also observed on the abaxial side of the base of older leaf primordia. In these cells, the difference between KN1 mRNA and protein localization is unlikely to be caused by differences in the stability of the gene products, because these cells left the meristem up to several days earlier. This proposed site of KN1 trafficking might also be biologically relevant. Sector analysis of maize shoot development suggests that cells in this position will form the axillary bud that is clonally associated with the leaf primordium (Johri and Coe, 1983; McDaniel and Poethig, 1988). The maintenance of KN1 protein in these cells could, therefore, play a role in the reactivation of meristem activity for the formation of an axillary meristem. In summary, the double labeling of KN1 gene products identified previously unknown potential sites of KN1 protein trafficking that may serve biologically relevant functions in shoot patterning.
A recent report using grafting experiments showed that a tomato KNOX fusion mRNA, encoded by the *Mouse ears* (*Me*) locus, is transported into a normal graft scion shoot, and this transport is correlated with a change in leaf morphology in the scion (Kim et al., 2001). The *Me* mutation is caused by a chromosomal rearrangement that results in a fusion of the 5′-coding and promoter region of *PYROPHOSPHATE-DEPENDENT PHOSPHFRUCTOKINASE*, which encodes a metabolic enzyme normally expressed in several tissues including the phloem, to most of the *LeT6* coding region (Chen et al., 1997). The localization of the *Me* transcript in the scion apex resembled that in nongrafted *Me* plants, implying that this specific pattern of transcript accumulation arises from spatial control of trafficking of the *Me* transcript, rather than from promoter activity. These observations of KNOX transcript trafficking agree with our earlier findings that KN1 can selectively transport its mRNA (Lucas et al., 1995); however, results presented here that show regions of the maize apex containing KN1 protein but not mRNA suggest that there must be tight spatial regulation of mRNA trafficking in the apex. Nonetheless, the remarkable discovery of long range KNOX transcript movement suggests that the long distance transport of regulatory mRNAs may control plant morphology and substantiate previous studies, which showed that many regulatory mRNAs are phloem mobile (Ruiz-Medrano et al., 1999).

Evidence for trafficking between cell layers in meristems was recently described for two other developmental transcription factor proteins, LEAFY (LFY) and SHORT-ROOT (SHR; Sessions et al., 2000; Nakajima et al., 2001). LFY is required for the inflorescence to floral transition, and mosaics of *FLORICAULA*, the snapdragon (*Antirrhinum majus*) ortholog of LFY, indicated that this gene acts nonautonomously to specify floral transition, and mosaics of *SHORT-ROOT* (*SHR*; Sessions et al., 2000; Nakajima et al., 2001). Therefore, SHR protein traffics from stele cells to the adjacent layer of cells and specifically accumulates in nuclei in these cells. In this case, trafficking appeared to be a specific property of the SHR protein, because there was no evidence for SHR mRNA transport, and the range of trafficking was only one cell layer, because no SHR protein was detected in the cell layer outside the endodermis. Therefore, for LFY and SHR, clear evidence has been presented for the biological significance of their cell to cell trafficking in meristems, supporting the hypothesis that KN1 trafficking in the meristem is also biologically relevant.

In conclusion, we have developed a double-labeling method that gives novel insights into possible sites of cell-to-cell trafficking of KN1. Our data, in combination with other recent reports of trafficking of mRNAs and proteins in meristems indicate that rather than acting like an open syncytium to allow free movement of gene products, meristems exert tight regulation over plasmodesmal trafficking to generate specific and developmentally significant protein and mRNA accumulation patterns.

**MATERIALS AND METHODS**

All solutions were made RNase free by treatment with diethyl pyrocarbonate, where appropriate (Maniatis et al., 1989). Median tissue pieces from the apical region, approximately 10×5×1-mm-thick, of 2-week-old maize (*Zea mays*) seedlings (873 inbred line) were dissected and vacuum infiltrated in FAA (formaldehyde-acetic acid-alcohol) (45% [v/v] ethanol, 5% [v/v] acetic acid, and 3.7% [v/v] formalin [Sigma, St. Louis]) for 5 to 15 min on ice, and then the fixative was renewed with fresh FAA, and formaldehyde-acetic acid and the tissue pieces were fixed, rotating at 4°C overnight. The apices that was single labeled in situ was from a tetraploid maize line. The tissues were then dehydrated, cleared, and embedded in wax (Paraplast Plus, Fisher Scientific, Loughborough, Leicestershire, UK), as described (Jackson, 1991). Ribbons of 10-μm sections were cut, and the meristem region was located using a dissecting microscope. The sections containing the meristem were flattened on water at 42°C on coated slides (Probe-on-Plus, Fisher Scientific), and the slides were dried overnight.

The sections were dewaxed, rehydrated (Jackson, 1991), and incubated for 5 min in phosphate buffered saline (PBS; 130 mM NaCl, 7 mM Na₂HPO₄, and 3 mM NaH₂PO₄, pH 7.3). They were then predigested for 10 min using 100 μg mL⁻¹ proteinase K (Sigma), rinsed three times in PBS for 2 min each rinse, and blocked in PBS containing 1 mg mL⁻¹ acetylated bovine serum albumin (BSA; Sigma) for 30 min. The blocking solution was drained off and
replaced with PBS-BSA containing affinity-purified KN1 antiserum (Smith et al., 1992) at a 1:10 dilution and 5 mg mL−1 heparin (Sigma), 100 µL per slide, and the slides were incubated in a humid rocking chamber for 2 h at room temperature. The heparin inactivates any RNAse activity present in the antiserum. Excess antibody was removed by rinsing in PBS-BSA three times for 10 min, and bound antibody was detected using a secondary antibody and an avidin-biotin-peroxidase complex (Vectorstain Elite kit, Vector Laboratories, Burlingame, CA) and DAB substrate for the peroxidase reporter (Vector Laboratories), according to manufacturer’s instructions. The sections were developed in DAB substrate, which generates an insoluble brown precipitate, for 5 to 15 min, and the reaction was stopped in water.

Next, the slides were blotted dry and blocked by prehybridization in hybridization (hyb) solution (Jackson, 1991) at 50°C for 4 h, using 250 µL of solution per pair of slides. The hyb solution was drained off and replaced with hyb solution containing digoxigenin-labeled antisense KN1 RNA probe, and hybridization, washing, and detection were as described (Jack-son et al., 1994). Slides were dehydrated and mounted in Cytoseal 60 (EM Science, Gibbstown, NJ) before microscopy.

Distribution of Materials

Upon request, all novel materials described in this publication will be made available in a timely manner for noncommercial research purposes, subject to the requisite permission from any third-party owners of all or parts of the material. Obtaining any permissions will be the responsibility of the requestor.

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