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RAPID COMMUNICATION

Tissue integrity and RNA quality of laser microdissected phloem of potato

YueYue Yu · C. C. Lashbrook · David J. Hannapel

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Abstract The phloem is an important conduit for the transport of signaling molecules including RNA. Phloem sap has served as a source of RNA to profile uncontaminated phloem transcriptomes but its collection is difficult in many species. Laser capture microdissection techniques offer a valuable alternative for isolating RNA from specific vascular cells. In potato (*Solanum tuberosum* L.), there are seven BEL1-like transcription factors expressed throughout the plant with diverse functions. The RNA of one of these, *StBEL5*, moves through the phloem from the leaf to stolon tips to regulate tuber formation. In this study, the presence of several *BEL* RNAs and one *Knotted1*-like RNA was determined in phloem cells collected by laser microdissection coupled to laser pressure catapulting (LMPC). Three fixatives were compared for their effect on cell morphology and RNA quality in transverse sections of stems of potato. For optimum tissue integrity and quality of RNA from potato stem sections, the best results were achieved using ethanol acetate as the fixative. In addition, the RT-PCR results demonstrated the presence of six out of seven of the *StBEL* RNAs and a potato *Knox* RNA in phloem cells.

Keywords Microdissection · Phloem · RNA · Plant stem · Tuberization · Vascular tissue

Y. Yu · D. J. Hannapel
Molecular, Cellular, and Developmental Biology Major,
Iowa State University, Ames, IA 50011, USA

Y. Yu
Department of Plant Pathology,
Iowa State University, Ames, IA 50011, USA

Y. Yu · C. C. Lashbrook · D. J. Hannapel (✉)
Department of Horticulture, Iowa State University,
Ames, IA 50011, USA
e-mail: djh@iastate.edu

Abbreviations

bp	Base pairs
LCM	Laser capture microdissection
LMPC	Laser microdissection coupled to laser pressure catapulting
LPC	Laser pressure catapulting

Introduction

In higher plants, the vascular system provides both mechanical strength and long-distance transport capacity. Vascular tissue is composed of three main units: xylem, cambium and phloem. The phloem distributes photo-assimilates from source to sink tissues and is composed of conducting sieve elements, associated companion cells, and nonconductive phloem parenchyma and fiber cells. In addition to carbohydrates, phloem transports hormones, mRNAs, and proteins that may mediate developmental and stress responses (Thompson and Schulz 1999; Citovsky and Zambryski 2000; Lucas et al. 2001; van Bel et al. 2002). Phloem is not merely a nutrient conduit, but also functions as an information highway (reviewed in Lough and Lucas 2006). Recent studies have implicated mobile RNAs in the regulation of flowering (Huang et al. 2005) and tuber formation (Banerjee et al. 2006a, b). If long-distance mobile molecules like mRNAs are being transported, it is very likely they can be detected in the phloem. Detection of RNAs in phloem cells, however, is not trivial due to the difficulty of access to the phloem cell contents and the abundance of enzymes that degrade RNA. In situ hybridization is a viable option but its level of detection is limited to moderately abundant transcripts.

Phloem sap collection has proven to be a valuable option with cucurbits (Ruiz-Medrano et al. 1999) but is not

practical for many other plant species (Pommerrenig et al. 2006). Anatomical features inherent in plants like celery (Vilaine et al. 2003) and common plantain (Pommerrenig et al. 2006) provide advantages for their use in the study of vascular tissue. Another useful approach for vascular tissue analysis has recently been developed that involves laser dissection of specific cells from tissue sections (Galbraith and Birnbaum 2006; Nelson et al. 2006; Day et al. 2007).

The earliest laser capture microdissection (LCM) techniques coupled dissection to the use of a thermoplastic transfer film to withdraw and collect target cells (Emmert-Buck et al. 1996). A more recent technique couples dissection to laser pressure catapulting (LMPC) (Bova et al. 2005). LMPC utilizes a pulsed ultraviolet-A laser to ablate specific cells viewed under a microscope. The laser beam is restricted to a tiny laser focal spot (<1 μm), leaving adjacent material fully intact (Micke et al. 2004). An intense defocused UV laser pulse just below the sample plane is then used to catapault the sample upward into the collection receptacle. Due to the high level of tissue organization and the stability of plant cell walls, LMPC is particularly useful for separating plant target cells from immediate neighbors. Certain cell types can be visually identified even from the unstained tissue sections and single cells can be ablated.

Optimization of microdissection methods for plants has required a multiplicity of approaches and techniques to meet disparate requirements imposed by specific tissues and species (Asano et al. 2002; Inada and Wildermuth 2005; Angeles et al. 2006). In this study, we assessed three fixative types for their ability to maintain structural quality of paraffin sectioned potato tissue and the effect of the fixative on RNA quality. LMPC was utilized to isolate vascular and nonvascular cells from which RNA was isolated. The presence of multiple *BEL1*-like mRNAs was determined by RT-PCR. The RNA for one identified BEL, *StBEL5*, is known to act as a long-distance mobile signal involved in short day-activated tuber formation (Banerjee et al. 2006b). The presence of additional mRNAs suggests that mobile signaling may be more complex than previously anticipated.

Materials and methods

Sample preparation

Cross-sections of stems from *Solanum tuberosum* ssp. *andigena* plants grown under short-days were trimmed to 5.0 mm in thickness. Segments were fixed 24 h at 4°C in 15 ml freshly prepared 3:1 (v/v) ethanol–acetic acid (Farmer's fixative), paraformaldehyde, or 100% acetone. Fixatives were infiltrated into the sections under vacuum (400 mm of Hg) for 15 min on ice. Fixed tissue was dehydrated at RT in a graded series of ethanol (1 h each 75, 85, 100, 100, 100%), followed by an ethanol/xylene series (1 h each 75:25%, 50:50%, 25:75% 0:100%, 0:100%, 0:100%). Flakes of Paraplast-XTra Tissue Embedding Medium (Fisher Scientific) were added to the final step. Once the flakes melted at RT, liquefied Paraplast-XTra was added, and sample vials were placed in the 60°C oven. The medium was replaced at 1.5 h intervals until residual xylene was absent. Samples were positioned in pure Paraplast-XTra, allowed to cool at RT and stored at 4°C. Sections were prepared using a rotary microtome (AO Spencer 820 Microtome, American Optical) to 10 μm thickness, floated in water on Probe-on positively charged microscope slides (Fisher Scientific) at 42°C to stretch the ribbons, air-dried at 42°C for 3–4 h, and stored at 4°C in a sealed slide box. Slides were used within 3–4 days. Just prior to microdissection, slides were deparaffinized twice for 10 min each in 100% xylene and air dried in the hood for 5 min. After LCM of cells from unstained sections, overall morphological quality was evaluated by staining mounted tissues with Safranin O/Fast Green.

Laser microdissection coupled to laser pressure catapulting (LMPC)

For microdissection, the PALM® Laser Microbeam instrument (Bernried, Germany) was employed. A pulsed UV nitrogen laser beam is projected through the objective lens to a narrow diameter (<1.0 micron) that ablates the target without heating adjacent material. Laser pressure catapulting (LPC), a high photonic pressure force, is used to capture the target cells into the lid of a LPC-microfuge tube. Cells were selected using the graphics tools of the PALM RoboSoftware. Specific cells from phloem, xylem and epidermis were catapulted separately into the lid of a 0.5 ml reaction tube (Zeiss, Hamburg, Germany) filled with 40 μl ethanol, For each sample an area of approximately 1.5×10^6 μm^2 comprised of approximately 5,000 cells was collected. Samples were visualized using a 20·XT objective (Leica Microsystems, Inc.).

RNA extraction and RT-PCR

To evaluate RNA quality, four tissue sections were removed with a scalpel blade from slides containing samples treated with one of three fixatives. Samples in the 40 μl ethanol from microdissected sections (Fig. 3), were collected by short-pulse centrifugation. RNA from all samples was isolated using the PicoPure® RNA Isolation kit (Arcturus, Mountain View, CA, USA). RNA was treated with the RNase-Free DNase Set kit (Qiagen, Valencia, CA,

USA) while in contact with the Picopure column membrane. Samples were incubated for 15 min at room temperature before eluting the column.

For the RNA quality test, RNA was reverse-transcribed using SuperScript™ III One Step RT-PCR System with Platinum® Taq DNA Polymerase Kit (Invitrogen), primed with 0.25 μM gene-specific primers (Table 1). RNA (3 ng) template were used with either the tubulin- or rRNA-specific primers (Table 1). PCR conditions were 50°C for 30 min; 94°C for 2 min; 29 cycles of 94°C for 30 s, 52°C for 30 s, 68°C for 1 min. This was followed by incubation at 68°C for 5 min before maintaining at 4°C after cycling. For the 18S rRNA, primers were provided from the manufacture's kit (Ambion QuantumRNA Universal 18S). The expected size of the rRNA product was 315 bp. PCR conditions were 50°C for 30 min; 94°C for 2 min; 22 cycles of 94°C for 30 s, 60°C for 30 s, 72°C for 30 s. This was followed by incubation at 72°C for 5 min before maintaining at 4°C after cycling.

For the StBEL and Knox target analysis, RNA was isolated from microdissected phloem cells of potato stem sections fixed by ethanol-acetic acid (Fig. 3). PCR conditions were adjusted based on the primer set used (Table 1). As a control, the samples were analyzed for the presence of the G2 phloem-specific mRNA of potato (TC118156). RNA was again subjected to RT-PCR using 0.25 μM gene-specific primers (Table 1).

Table 1 Gene-specific primers used for RT-PCR reactions with expected product size

Primer	Sequence (5′⇒3′)	Length of PCR product (bp)
TubF	CTGGGAAGTTATCTGTGATG	605
TubR	GTAGTGAGCTTCAAAGTCCT	
G2F	ACAACCGCACAAAGAATTTAATG	400
G2R	TGTTCTCCACATATGTTCAAAT	
BEL5F	ATACCAGAAAGTCTCG	310
BEL5R	AATACTACTAGTTGTATCAAT	
BEL14F	ACAACATGGTGGAAGTG	193
BEL14R	CCAGCCAAATCATGAAG	
StBEL11F	GCTTCCACCAAATTCTG	133
StBEL11R	TAGTTGAGTGGCGTATC	
StBEL13F	CTGCCACTCCATAACTG	264
StBEL13R	TTCCTGCATG TCGTAACC	
StBEL22F	CTTCCTCTATGCCAACTC	186
StBEL22R	GCAGTCCTAAGGTAAGTG	
StBEL29F	ATGATAGGCAGGACAGAG	305
StBEL29R	GACGCTTGATGTTGAGTG	
StBEL30F	CAGGACGCATTTG TTCAAAG	211
StBEL30R	TTTCAGGGACCATAGTAGTAGG	
POTH1F	GTTGTGGTGAGGCAGATG	210
POTH1R	TCTTATCAGCCTC CGTAG	

Tub Tubulin, *G2* a phloem-specific transcription factor (Zhao et al. 2005), were used as controls, *POTH1* is a *knotted1*-like gene of potato (Rosin et al. 2003)

Results

As a prelude to LMPC (Niyaz et al. 2005), the effect of chemical fixatives on cell morphology of transverse stem sections was compared (Fig. 1). Cells from the sections prepared with acetone fixative appeared compacted and distorted. Most compaction was evident in the vascular cambium and phloem cells (arrow, Fig. 1a). Xylem cells also appeared to be collapsed (Fig. 1a, X). Xylem and phloem cells from sections prepared with the ethanol–acetic acid fixative displayed very little compaction or degradation (arrows, Fig. 1b). Formaldehyde-fixed sections exhibited the best morphological quality with cells that displayed very little distortion or compaction (Fig. 1c).

RNA was isolated from four fragments scraped from sections prepared with each fixative and yields for the sections prepared with formaldehyde, ethanol–acetic acid, or acetone were 10, 31, and 92 ng, respectively. To assess quality, RNA was used in RT-PCR reactions with primers specific for 18S ribosomal or tubulin RNA. The PCR reactions were monitored to determine conditions under which synthesis of the product was in the linear range. RNA from the acetone sections produced the highest yield of PCR product for both tubulin and rRNA (Fig. 2; A samples), whereas no product was detected under these conditions with the formaldehyde sample (P samples). Relative to the acetone product, a lower level of product was observed for the ethanol-acetate sample for both tubulin and rRNA (Fig. 2; E samples). In considering both the integrity of cell morphology and the quality of the RNA sample, sections prepared with ethanol–acetate were used for further microdissection analyses.

Selected cells were microdissected from regions of the phloem (Fig. 3a–b), xylem (Fig. 3c–d) or epidermis plus some of the collenchyma cells (Fig. 3e–f). RNA extracted from cells served as the template for RT-PCR using gene-specific primers. The G2 control specific for phloem cells serves as a negative control for xylem and epidermal, whereas tubulin serves as a positive control. *StBEL5* and *-14* were tested in RNA from xylem, phloem and epidermal cells in previous work (Banerjee et al. 2006b). RNA of *StBEL5* was present in all three, whereas RNA of *StBEL14* was detected only in epidermal cells at very low levels.

RT-PCR tested for the presence in phloem of all seven of the RNAs of the potato BEL1 family (Chen et al. 2003) and POTH1, the protein partner of StBEL5 (Rosin et al. 2003). Six of the 7 *BEL1*-like RNAs and the *POTH1* RNA

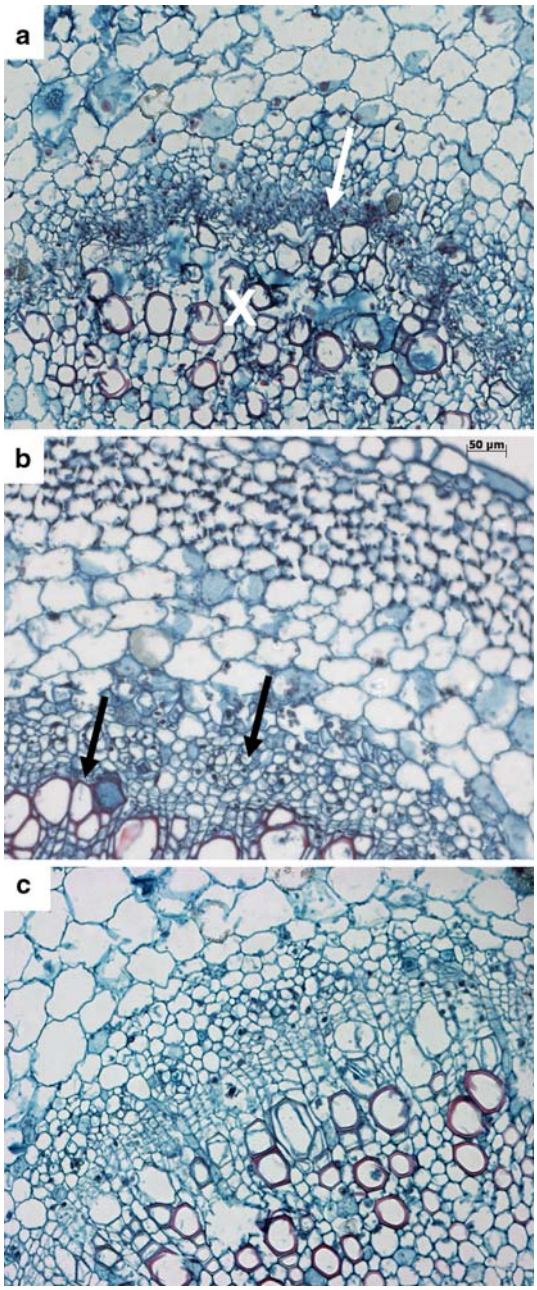

Fig. 1 Transverse section of potato stems using different fixatives: 100% acetone (**a**), ethanol–acetic acid (3:1) (**b**), paraformaldehyde (**c**). Sections were stained with Safranin O/Fast Green to highlight cell walls. (*X* xylem)

were detected in RNA extracted from phloem cells (Fig. 4). These PCR reactions were performed with relatively low-stringent conditions to improve the likelihood of detection of any *StBEL* transcripts in the RNA from phloem cells. All bands of the expected size were confirmed via sequencing. The extra bands that appear are non-specific PCR product that routinely occurs under these conditions. In agreement with prior results, *StBEL14* was not detected in phloem cells (Banerjee et al. 2006b). These data verify the

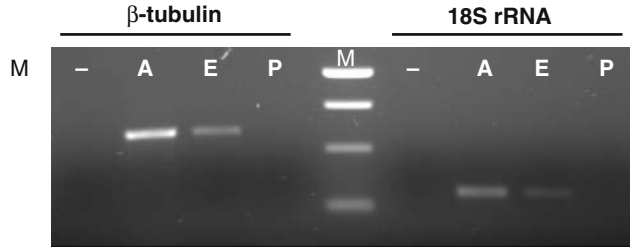

Fig. 2 RT-PCR of RNA extracted from transverse stem sections of potato stems using either 100% acetone (*A*), ethanol-acetic acid, 3:1 (*E*), or paraformaldehyde (*P*) as a tissue fixative. Equal volumes of the RT-PCR reaction were loaded for each lane. RNA-specific primers used for the PCR reactions are listed in Table 1

effectiveness of this protocol for the identification of diverse RNAs present in specific potato cells.

Discussion

Sample preparation is critical to insure quality of morphology and RNA integrity

The importance of the fixative on both cell integrity and resulting RNA quality is not surprising. In plant microdissection studies, there is considerable variation in fixative treatments for different species and the organ type. Ohtsu et al. (2007) used Farmer's fixative and embedded samples in paraffin to analyze the roots of a 3-day-old seedling. For their analysis of RNAs in rice phloem cells, Asano et al. (2002) used a modified LCM system with tissue fixed in 100% acetone. Angeles et al. (2006) used the same PALM system reported here for their isolation of stem cells from *Urtica diocia* L. Optimum results were achieved by using a fixative with a mixture of 0.2% glutaraldehyde and 2% formaldehyde in 0.05 M phosphate buffer (pH 7.0–7.2). Similar to our approach, Ivashikina et al. (2003) effectively used Farmer's fixative and paraffin-embedded sections for patch-clamp studies of phloem cells from inflorescence stalks of *Arabidopsis*.

Using LCM of *Arabidopsis* silique tissues from tape-transferred sections, Cai and Lashbrook (2006) established paraffin embedding (Kerk et al. 2003) and an ethanol/acetic acid fixative (EAA) as the methods of choice for supporting gene-profiling studies. They showed that the integrity of RNA was compromised during the stretching of paraffin ribbons onto water or slide drying and that the use of a tape-transfer system obviates this problem. Both Wu et al. (2006) and Nakazono et al. (2003) used Farmer's fixative combined with a supplementary sucrose infiltration to minimize the formation of ice crystals during cryosectioning of cotton ovaries and maize coleoptiles, respectively. Both techniques used the PALM laser capture system and

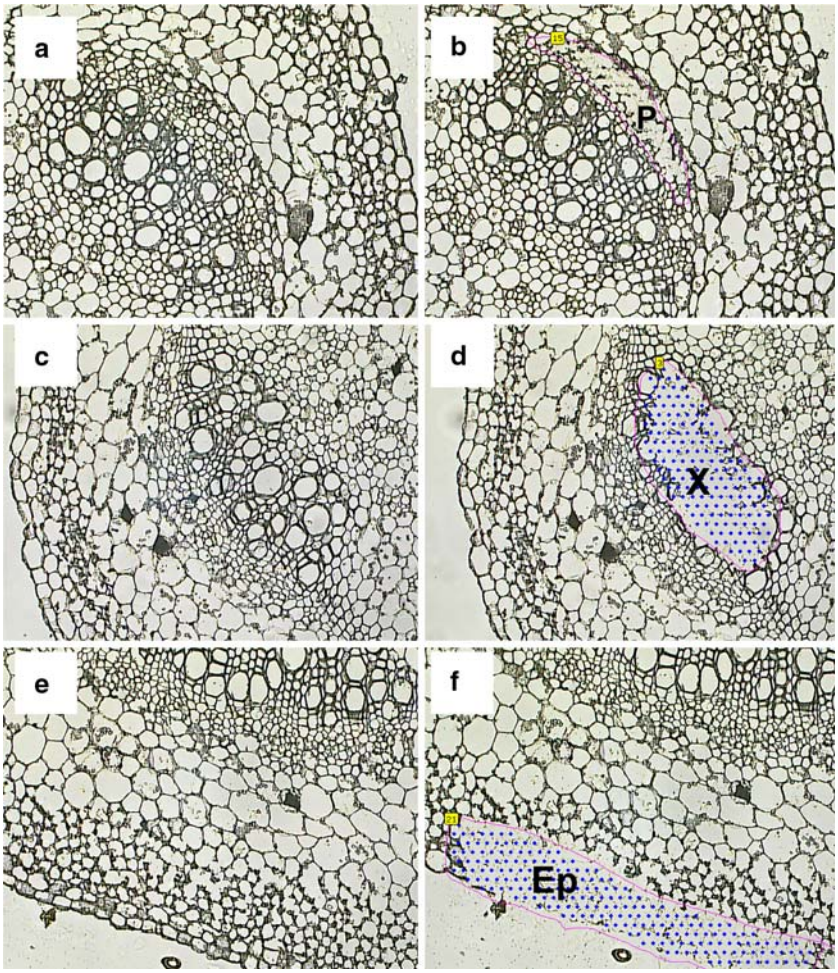

Fig. 3 LMPC of different tissue types: Phloem cells (**a**, **b**), xylem cells (**c**, **d**), epidermal cells with the collenchyma cells (**e**, **f**). Sections before LMPC (**a**, **c**, **e**), sections after LMPC (**b**, **d**, **f**). (*X* xylem; *P* phloem; *Ep* epidermal). The *dotted areas* indicate the site of laser excision

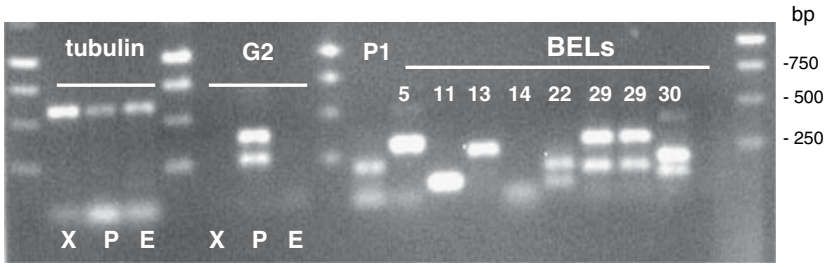

Fig. 4 Identification of specific RNAs from potato stem phloem cells using LMPC/RT-PCR. Selected cells from xylem (*X*), phloem (*P*) or epidermis plus some collenchyma cells (*E*) were excised by focused laser light and collected via catapulting with pressure into the collection tube. Total RNA was extracted from LMPC-collected cells and used as the RT-PCR template for detecting tissue-specific transcripts. Gene-specific primers (Table 1) for the potato Knox RNA, *POTH1* (*P1*), and seven *StBEL* RNAs (designated *5*, *11*, *13*, *14*, *22*, *29*, and *30*) were tested for their presence in phloem RNA. *G2* is RNA for a phloem-specific transcription factor (Zhao et al. 2005). Tubulin primers were used with the various RNAs as a positive control. The identity of specific bands for each product was verified by sequencing. Source plants for these experiments were grown under short-day conditions (8 h light, 16 h dark). Size markers (bp) are shown on the far right

sections were embedded in TissueTek OCT medium. RNA was extracted, amplified and used successfully in microarray analysis.

BEL1-like RNAs and *POTH1* are present in the phloem

The presence of so many *StBEL* RNAs in the microdissected phloem cells of the stem was unexpected. To date only *StBEL5* RNA is known to function as a long-distance signal that moves through phloem (Banerjee et al. 2006b). In this report, all the *StBEL* RNAs were detected in phloem cells except *StBEL14* (Fig. 4). In previous work (Banerjee et al. 2006b), a wide range of transcript abundance for *StBEL5* (present in xylem, phloem, and epidermal cells) and *StBEL14* (low levels in epidermal cells only) has been verified using this technique. The seven BELs of potato

exhibit diverse RNA accumulation patterns (Chen et al. 2003) and this may reflect their different biological functions and movement patterns. *BEL1*-like genes are known to function in floral development (Bhatt et al. 2004; Smith et al. 2004; Kanrar et al. 2006). The RNAs of *StBEL13* and *-30*, for example, are very abundant in shoot apices and flowers but barely detected in potato tubers (Chen et al. 2003).

The presence of the RNA for the Knox protein of potato, POTH1, in the phloem (Fig. 4) was also unexpected. Previous work has established POTH1 as the protein partner of StBEL5 in a transcriptional complex that represses activity of the *GA20 oxidase1* gene (Chen et al. 2004). Long-distance movement of a *Knox* RNA was demonstrated (Kim et al. 2001) but very little is known about the putative function of this RNA mobility. The fact that two RNAs that encode for interacting proteins are moving through the phloem simultaneously is intriguing. BEL and Knox proteins are ubiquitous in the plant kingdom and regulate numerous developmental processes (Hake et al. 2004).

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