Identification of StBEL5 RNA as a long-distance mobile signal in short-day facilitated tuber formation

Yueyue Yu
Iowa State University

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Identification of *StBEL5* RNA as a long-distance mobile signal in short-day facilitated tuber formation

by

Yueyue Yu

A thesis submitted to the graduate faculty
in partial fulfillment of the requirements for the degree of

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Program of Study Committee:
W. Allen Miller, Major Professor
  David J. Hannapel
  Gustavo Macintosh

Iowa State University
Ames, Iowa
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ABSTRACT

In potato, the BEL1-like transcription factor, StBEL5 and its protein partner POTH1, regulate tuber formation by mediating hormone levels in the stolon tip. Heterografting experiments show that StBEL5 mRNA can move across the graft union to localize in stolon tips and enhance tuber formation. Over-expression of StBEL5 full-length transcripts including the untranslated regions (UTRs) endows transgenic lines with the capacity to overcome the long-day inhibitory effects on tuber formation.

In this study, the precise localization of endogenous StBEL5 mRNA and other gene specific transcripts in the vascular tissues were determined by laser microdissection coupled to laser pressure catapulting (LMPC) and following RT-PCR. The results demonstrate the presence of StBEL5 mRNA in phloem cells which is consistent with its role as a mobile RNA.

StBEL5 full length transcripts exhibited better mobility compared to its UTR-truncated form and StBEL14 transcripts in agroinfiltration experiments. No translation enhancement was observed for full length StBEL5 transcripts compared to its UTR-truncated transcripts by in vitro translation assay. This indicates involvement of StBEL5 UTRs in facilitating its long-distance movement. Further studies of applying EMSA and northwestern blotting to identify StBEL5 mRNA binding proteins may provide pivotal cues to understand the mechanism of phloem delivered StBEL5 RNA in this long-distance signaling pathway during tuber formation.
CHAPTER 1. GENERAL INTRODUCTION

Potato is one of the most important crops worldwide with annual production approaching 300 million tons, ranking fourth after three cereals: maize, rice and wheat. Therefore, extensive research has been directed at improving the yield and quality of potatoes, increasing the resistance to pathogen infection and producing tubers with enhanced nutritional qualities. Besides the great economic importance, research on potatoes has many advantages: they can be easily propagated by vegetative means and are accessible to transgenic manipulations (Jackson, 1999; Banerjee et al., 2006, 2007).

Potato tubers develop from stolons which are actually modified underground stems. Under noninductive conditions, the stolons grow horizontally or sometimes grow upwards, emerging from the soil to form new shoots. Under conditions favoring tuberization, the elongation of the stolon tip ceases, and the subapical part of the stolon swells to form tubers. There are many environmental and endogenous factors that influence tuber formation. Temperature, photoperiod, nitrogen level, sucrose concentration, and several plant hormones are all involved in this coordinated process (see reviews: Jackson, 1999; Martinez-Garcia et al., 2001; Hannapel et al., 2004).

For certain short-day (SD) adapted potato species such as Solanum tuberosum ssp. andigena, SD photoperiods are strictly required for tuber formation and this subspecies does not tuberize when grown under long-day (LD) conditions or SD supplemented with a night-break. Due to its strict sensitivity to photoperiod, andigena plants have been widely used in photoperiod signaling studies. Because leaves are the
photoperiod perception sites, not the stolons, it has been hypothesized that a substance is synthesized in the leaves under SD conditions and transported to the stolons, where it triggers tuber formation.

The existence of such transmittable signals was confirmed by grafting experiments. When the scions of SD-grown *andigena* plants were grafted onto the noninduced stocks, tuberization was observed under LD treatment (Gregory, 1956; Kumar and Wareing, 1974). Furthermore, interspecific grafting experiments between potato and tobacco show that flowering tobacco grafted onto *andigena* potato stock grown under LD can induce tuber formation (Chailakyan et al., 1981). This result suggests tuberization in potato and flowering in tobacco may be mediated by the same phloem-transported signals. Although the precise nature of such transmittable signals is still poorly understood, several genes have been identified that are involved in photoperiod regulation on tuberization. A transgenic study showed *phytochrome B* (PHYB) regulates a transmissible inhibitor of tuberization (Jackson et al., 1998). Recently, functions of some potato orthologs of *CONSTANS (CO)*, *GIGANTEA (GI)* and *FLOWERING LOCUS T (FT)* proteins have been identified in controlling tuberization under SD (Martínez-García et al., 2002; Rodríguez-Falcón et al., 2006).

In *Arabidopsis*, CO and FT are involved in controlling flowering time in a photoperiod-mediated pathway. CO expression is under photoperiod control in companion cells of the source leaves where it activates FT expression. FT mRNA has the capacity to move long distance via the phloem from the leaf to the shoot apical meristem (SAM) where the FT protein can interact with a bZIP transcription factor
FD. The complex of FT and FD proteins can activate floral identity genes triggering the SAM to inflorescence meristem (IM) transition (Huang et al., 2005; Wigge et al., 2005). This breakthrough discovery again leads to the hypothesis that a similar long-distance signaling exists in the phloem that controls the photoperiod-mediated tuberization in potato.

A variety of macromolecules has been reported to travel through the phloem, including cellular mRNAs (Kim et al., 2001; Ruiz-Medrano et al., 2001); pathogenic RNAs (Carrington et al., 1996; Heinlein, 2002; Zhu et al., 2001), small RNAs, putative components of the gene silencing phenomenon (Mlotshwa et al., 2002; Yoo et al. 2004) and numerous proteins as well, such as chaperone protein (Aoki et al., 2002), proteinase inhibitors (Haebel and Kehr, 2001), RNA-binding proteins (Jorgensen et al., 1998; Xoconostle-Cázares et al., 1999). Delivery of these macromolecules to distant tissues might reflect a mechanism used by plants to regulate developmental and defense processes at the whole plant level. (see review: Lough and Lucas 2006).

Studies on the phloem transported long-distance mobile RNAs controlling the leaf shape provide strong evidence for the functional significance of such RNA movement. In tomato, when wild type scions were grafted onto a naturally occurring dominant KNOX mutants, the mutant transcripts (PFP-LeT6 fusion transcripts) were able to move to the wild type scions and resulted in the a modified leaf phenotype (Kim et al., 2001). In Arabidopsis, it was reported KNOTTED1 (KNI) RNA binds to its protein to facilitate cell-to-cell movement (Kim et al., 2005).
The *knox* (knotted-like homeobox) gene belongs to the TALE (three amino acid loop extension) superclass of transcription factors. Two major groups of plant proteins are found in the TALE family, KNOX and BEL-like (Bürglin, 1997). The interaction of KNOX and BEL proteins was documented in barley, *Arabidopsis*, maize and potato (Müller *et al.*, 2001; Bellaoui *et al.*, 2001; Smith *et al.*, 2002; Chen *et al.*, 2003). The KNOX protein of potato, designated POTH1, regulates plant growth by controlling gibberellic acid (GA) synthesis and enhancing cytokinin levels (Rosin *et al.*, 2003).

Using POTH1 as bait, seven distinct BEL1-like proteins of potato were identified by yeast two hybrid screening (StBEL5, 11, 13, 14, 22, 29, 30) (Chen *et al.*, 2003). StBEL5, one of the potato BELs, exhibited increased RNA accumulation levels in response to SD condition. Enhanced tuber formation and increased cytokinin levels were also observed in *StBEL5* over-expression lines, similar to the *POTH1* over-expression transgenic lines (Chen *et al.*, 2003). Transcription assays with the BEL and KNOX proteins of potato indicate that the BEL-KNOX heterodimeric complex is required for repression of promoter activity of *ga20 oxidase1* (Chen *et al.*, 2004). This repression is correlated with the reduced GA level in the stolon tip when tuberization occurs.

*StBEL5* RNA is ubiquitous in potato plants and its accumulation is enhanced in leaves and stolons under SD conditions (Chen *et al.*, 2003). Other than the effect on tuber formation, these *StBEL5* over-expression lines exhibited similar phenotypes to wild type plants. Based on *StBEL5* RNA localization and accumulation patterns, we
hypothesize that \textit{StBEL5} RNA acts as a long-distance mobile signal involved in the SD facilitate tuber formation (Banerjee \textit{et al.}, 2007).

To test our hypothesis, \textbf{laser} microdissection coupled to \textbf{laser pressure} catapulting (LMPC) was applied to collect tissue-specific samples from transverse sections of wild type potato stems. The precise localization of \textit{StBEL5} mRNA and other gene specific transcripts in the vascular tissues were then determined by RT-PCR using RNA samples isolated from LMPC harvested cells. The mobility of full length \textit{StBEL5} mRNA was compared with the truncated \textit{StBEL5} transcripts and \textit{StBEL14} mRNA using an agroinfiltration method. Furthermore, the function of the \textbf{untranslated} regions (UTRs) of \textit{StBEL5} mRNA was analyzed by an in vitro translation assay.
CHAPTER 2. IDENTIFICATION AND COMPARISON OF PHLOEM LOCALIZED STBELS BY LMPC

Introduction

In higher plants, the vascular system provides both mechanical strength and long-distance transport of water, nutrients and signaling compounds to the whole plant. Vascular tissue is composed of three main units: xylem, cambium and phloem. The cambial zone lies between xylem and phloem and gives rise to xylem and phloem cells. The xylem is the main conduit for water and mineral nutrients that travel from the root to leaf. It also transports hormones such as abscisic acid and cytokinin (Haberer and Kieber, 2002; Hartung et al., 2002). Xylem is composed of water-conducting tracheary elements and nonconducting parenchyma and fiber cells. The phloem distributes photoassimilates from source to sink tissues. It is composed of conducting sieve elements (SEs), associated companion cells (CCs) and nonconducting phloem parenchyma and fiber cells.

Recent evidence has shown that besides photosynthetic products, hormones, mRNAs, and proteins are also transported via the phloem. Some of the long-distance transported molecules play important roles in plant development and responses to biotic or abiotic stresses (Thompson and Schulz, 1999; Citovsky and Zambryski, 2000; Lucas et al., 2001; van Bel et al., 2002). These studies indicate phloem is not merely a nutrient conduit, but also functions as an information pathway. If long-distance mobile molecules like mRNAs are being transported, it is very likely they can be detected in the phloem. However, detection of mobile RNAs in phloem translocation
tubes is not easy due to the difficulty of access to the phloem cell contents.

Several studies have made use of the collected phloem sap to isolate phloem proteins and RNAs in wheat, rice, castor bean and cucurbits (Hayashi et al., 2000). However potential contamination of other non-vascular cells that are damaged from the cut surface may occur.

Aphid styletomy is another elegant technique to obtain phloem-enriched samples. Phloem feeding insects such as aphids are placed on the plant, the stylets are severed from the insects after feeding, phloem sap then can be collected when it exudes from the stump of stylets (Doering-Saad et al., 2002). This technique leads to small pressure changes and has potential local contamination too. So the accuracy of the analysis to identify phloem localized molecules is dependent upon the collection technique.

LMPC is a useful tool for collecting tissue-specific samples or morphologically defined cell populations from plant tissue sections. LMPC utilizes a pulsed Ultraviolet A (UVA) laser which is focused through the microscope to allow laser ablation of cells on a tissue section. Such laser beam is restricted to a tiny laser focal spot (<1μm), leaving adjacent material fully intact (Mieke et al., 2004). Then an intense defocused UV laser pulse just below the sample plane is used to propel the sample upward and catapult it into the collecting microfuge cap. Cells isolated by LMPC can be used in cell-specific DNA, RNA, protein or metabolic profiling studies (Nelson et al., 2006). Due to the highly regulated tissue organization and the stable cell walls of plants, LMPC is particularly applicable to plant tissue sections. Certain
cell types can be visually identified even from the unstained tissue samples.

By exploiting LMPC in this study, we successfully isolated xylem, phloem, and nonvascular tissue cells from potato stem transverse sections. The following comparative analysis of tissue specific transcripts using RT-PCR not only demonstrates the presence of \textit{StBEL5} mRNA in phloem cells which is consistent with its role as a mobile RNA, but also indicates the specific functions of different \textit{StBELs} in different tissue development. \textit{StBEL14} is the only \textit{StBEL} gene whose mRNA is not presented in the phloem but in the epidermis. This may imply its role in epidermis development which originates from the cells of shoot apical meristem (SAM) L1 layer.

\textbf{Materials and Methods}

\textit{Sample Preparation}

\textit{Solanum tuberosum ssp. andigena} (line 7540) plants were grown in soil in the greenhouse until they reached 10~15 leaf stage, and then transferred to growth chamber under SD condition (8 h light and 16 h dark, 20°C) or LD condition (8 h dark and 16 h light, 20°C) for 10 days.

\textit{Fixation}

Cross-sections of the stem samples were trimmed to 5mm in thickness and fixed 24 h at 4°C in 15 mL freshly prepared 3:1 (v/v) ethanol-acetic acid (Farmer’s fixative). This fixative was infiltrated into the sections under vacuum (400 mm of Hg) for 15 min on ice. Two other fixatives: paraformaldehyde and 100% acetone were also tested. Ethanol-acetic acid was the best fixative to provide the acceptable RNA
recovery and good morphological preservation (Figure 1B). Fixed tissue was dehydrated at room temperature 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 room temperature, 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 any residual of xylene was absent. Samples were positioned in pure Paraplast-XTra, and sections were cut on a rotary microtome (AO spencer 820 Microtom, American Optical) to 10 μm thick, floated in water on Probe-on positive charged microscope slides at 42°C to stretch the ribbons, air-dried, and stored.

Deparaffinization

Slides were deparaffinized twice for 10 min each in 100% xylene. Then air dry slides in hood for 5 min. Sections were deparaffinized just prior to laser microdissection.

LMPC

For microdissection, the PALM® Laser Microbeam instrument (Bernried, Germany) was employed. This system consists of a low heat UV (337 nm nitrogen) laser and an inverted microscope. A pulsed UV nitrogen laser beam is first focused through the objective lens to a less than 1.0 micrometer diameter beam spot that ablates the target without heating adjacent material. Then, 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. After selection, specific cells from phloem, xylem and epidermis were isolated separately by the laser microbeam and collected by LPC into the lid of a 0.5 ml reaction tube (Zeiss, Hamburg, Germany) filled with 40 μl ethanol, placed in a holder closely above the slide. For each sample an equal area of approximately $1.5 \times 10^6 \mu m^2$ was collected.

**RNA isolation**

RNA from the ethanol/acetic acid-fixed cells is extracted and isolated using the PicoPure® RNA Isolation kit and protocol (Arcturus, Mountain view, CA). Picopure isolated samples were treated with RNase-Free DNase Set kit (Qiagen, Valencia, CA) according to the manufacturer while samples were on the Picopure column membrane, incubating for 15 min at room temperature before eluting the column.

**RT-PCR**

RNA was isolated from phloem, xylem and epidermal cells from potato stem sections. To evaluate the purity of cell types, we analyzed the samples by RT-PCR for the presence of the specific mRNA from a potato phloem gene (TC118156), similar to the G2-like transcription factor gene in *Arabidopsis* as a positive control (Zhao *et al.*, 2005); and mRNA from a specific potato root gene (CK267169) homologue to *Arabidopsis* high affinity nitrate transporter gene *NT* as a negative control. This marker gene has been demonstrated by in situ hybridization and reporter gene expression (Nazoa *et al.*, 2003).
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 primer. The PCR conditions were adjusted based on the primers used. Primers were as follows:

G2-F: 5' ACAACCGCACAAAGAATTTAATG 3'
G2-R: 5' TGGTCTCCACATATGGTCAAT 3' (400bp)
NT-F: 5' TGTTCTCCACATATGGTCAAT 3'
NT-R: 5' TGGTCTCCACATATGGTCAAT 3' (400bp)
BEL3UTRa-F: 5' ATACCAGAAAGTCTCG 3'
BEL3UTRa-R: 5' ATACCAGAAAGTCTCG 3'
BEL14-F: 5' ACAACATGGTGGAAGTG 3'
BEL14-R: 5' ACAACATGGTGGAAGTG 3'

Results

To increase the sensitivity of detecting specific RNAs in a defined number of homogeneous cells derived from stems, we made use of LMPC (Niyaz et al., 2005) from transverse paraffin sections of wild type potato stems on the PALM Microlaser system (Plant Sciences Institute, Iowa State University). For microdissection, focused laser light was used to excise selected cells from regions of the phloem (Figure 2A), xylem (Figure 2C) or epidermis plus some of the collenchyma cells (Figure 2E). After microdissection, the sample cells were directly catapulted into an appropriate collection device (Figure 2B, D, F). RNA was extracted from these cells and used as
the template for RT-PCR using gene-specific primers. Identity of PCR products was confirmed by sequencing isolated bands. Data shown in Figure 3 was from SD grown plants. Identical results were also obtained when using material from LD-grown plants.

RT-PCR of RNA extracted from specific stem cells of both LD- and SD-grown plants showed that \textit{StBEL5} RNA was present in xylem, phloem, and epidermal cells (Figure 3B). All seven of the RNAs of the BEL1 family from potato (Chen \textit{et al.}, 2003) were tested and only \textit{StBEL14} RNA was not detected in phloem cells, but in the non-vascular tissues (Figure 3B, C). Previously, the RNA from \textit{StBEL14} was detected in flowers, leaves and roots (Chen \textit{et al.}, 2003). RNA for the protein partner of StBEL5, POTH1, was also detected in phloem cells (Figure 3C). RNA from a nitrate transporter NT, specific to roots (Nazoa \textit{et al.}, 2003) was used as a negative control and the RNA for a G2-like transcription factor, specific to phloem cells (Zhao \textit{et al.}, 2005), was used as a positive control (Figure 3B). The results of the LMPC/RT-PCR analysis verify the presence of \textit{StBEL5} RNA in phloem cells of potato stems. Consistent with the LMPC results, the presence of \textit{StBEL5} RNA in the phloem was confirmed by in situ hybridization data (Banerjee \textit{et al.}, 2007).

**Discussion**

LMPC has been established as an efficient cell-specific harvesting method for plants and animals. Compared with other techniques such as direct micropipetting of cell contents (Brandt \textit{et al.}, 2002; Raps \textit{et al.}, 2001), fluorescence activated sorting of protoplasts from dissociated tissues (Birnbaum \textit{et al.}, 2003; Nawy \textit{et al.}, 2005),
tissue homogenization and cell fractionation (Sheen and Bogorad, 1987) and hand
microdissection (Schrader et al., 2004; Outlaw and Zhang, 2001), LMPC is more
advantageous because it allows contamination-free collection of large homogeneous
cell population from plant tissue sections that can be viewed by microscopy. Even the
limited amount of RNA that can be recovered by LMPC is becoming less of the
problem, as methods for RNA amplification are currently well established.

Tissues fixation is a critical step in sample preparation during LMPC, a
balance between morphological preservation and RNA recovery should be achieved.
After a successful fixation, the target cells should be easily distinguished from the
neighboring cells, and the DNA, RNA or proteins to be extracted from the target cells
should be well preserved. The cross-linking fixative paraformaldehyde provided the
best histological detail (Figure 1A), but less RNAs can be extracted. The
noncross-linking fixative 100% acetone can provide the highest RNA preservation
capacity, but the cell morphology after the fixation is poor (Figure 1C). In this LMPC
experiment, the ethanol-acetic acid fixative was selected because it provided the
acceptable histological detail of the target cells (Figure 1B) and quality preservation
of RNA from the tissue sections. The RNA yield was compared using one whole
piece of section scratched from the slides with samples fixed in different fixatives.

Promoter activity of StBEL5 was detected in leaf veins, leaf petioles, stolons
and newly formed tubers. Transverse sections of the petiole exhibited the greatest level
of StBEL5 promoter activity in vascular cells (Banerjee et al., 2007). Together, these
data suggest that StBEL5 mRNA is transported through the phloem.
Presence of StBEL5 RNA in the microdissected xylem and epidermal cells of the stem was unexpected. It is plausible that StBEL5 RNA in the phloem could move cell-to-cell to adjacent regions like the xylem and epidermis. The external phloem of potato is situated between these areas (Figure 3A). Cell-to-cell movement has been observed for Knotted1 RNA in the SAM (Lucas et al., 1995) and in mesophyll and epidermal cells of leaves (Kim et al., 2005). During xylem differentiation, secondary cell walls are deposited after plant cells stop expanding. Generally they are composed of a complex mixture of lignin, cellulose/hemicellulose, proteins and other minor components (Gibeaut and Carpita, 1994). The lignin biosynthetic pathways are known to be negatively regulated by KNOX proteins (Mele et al., 2003; Hake et al., 2004). StBEL5, along with other Knox partners of potato, could be involved in a similar function. It is well established that xylem and phloem cells are interconnected and are formed in close relationship with each other (Lalonde et al., 2003). Based on transcription profiles, numerous transcription factors, like Dof and NAC proteins, are represented in both xylem and phloem cells and likely perform roles in vascular tissue development (Zhao et al., 2005).

An interesting discovery in this LMPC experiment is that all the StBEL RNAs were detected in the phloem cells except StBEL14 (Figure 3C). That the RNA of StBEL14 is absent in the vascular tissues but present in the epidermis indicates that StBEL14 is not mobile in the phloem under these conditions. StBEL14 RNA is most abundant in flower, leaf and root, but not in the tuber and stem according to the northern-blotting results (Chen et al., 2003). Whereas StBEL5 RNA accumulation is
ubiquitous. These different RNA accumulation patterns may reflect their different biological functions. BEL1-like genes are known to function in floral development (Bhatt et al., 2004; Smith et al., 2004; Kanrar et al., 2006). StBEL RNAs could be moving into floral meristems to regulate inflorescence architecture or to affect the competence to respond.

Future application of LMPC in this mobile signaling study may include phloem transcriptome comparison of the SD and LD growing potatoes, or potato phloem cDNA library construction. cDNA libraries have been constructed for the phloem RNAs from rice using LMPC (Asona, et al., 2002).
CHAPTER 3: MOVEMENT COMPARISON OF SELECTED STBEL RNAs USING AN AGROBACTERIUM-MEDIATED INFILTRATION METHOD

Introduction

Agroinfiltration is a transient gene expression method based on the Agrobacterium-mediated T-DNA transfer mechanism. The gene of interest is first cloned into the T-DNA region of the binary vector, and then transferred into a suitable Agrobacterium strain. The recombinant Agrobacterium culture is vacuum-infiltrated into the intact leaves. And upon the bacteria penetrating into the intercellular spaces of leaf parenchyma, and transferring its T-DNA into the nucleus, the non-integrated T-DNA can be transiently maintained and transcribed in the nucleus, leading to the expression of the foreign gene (Kapila et al., 1997).

The benefit of agroinfiltration compared to traditional plant transformation is speed and convenience. Transient expression occurring in the majority of cells within the infiltrated regions often exceeded those obtained in stably transformed plants (Vaquero et al., 2002). The amount of tissue transiently expressing a gene is sufficient to perform RNA or protein analysis. For genes that have deleterious effects on plant growth and development, agroinfiltration also provides a rapid way to assay the function of those genes (Wroblewski et al., 2005).

Compared with other transient expression systems such as particle bombardment and protoplast transformation, agroinfiltration is much easier to perform and applicable to study tissue-specific or developmentally regulated gene expression and processes involving interaction between neighboring cells. Another
advantage of agroinfiltration is that several foreign genes can be co-expressed in the
same cells by infiltrating the mixture of different *Agrobacterium* cultures.

Agroinfiltration has been widely used in transgenic complementation study
(Johansen and Carrington, 2001; Shao et al., 2003), promoter analysis (Yang et al.,
2000), selected protein production (Vaquero et al., 1999, 2002) and
post-transcriptional gene silencing (Llave et al., 2000; Kościańska et al., 2005).

The most popular host plant for agroinfiltration is *Nicotiana benthamiana.*
This technique has also been described for other species like *Medicago sativum*
(D’Aoust et al., 2004), lettuce, tomato (Orzaez et al., 2006), and *Arabidopsis*
(Wroblewski et al., 2005). Considering *Nicotiana benthamiana* and *Solanum
tuberosum* are from the same *Solanaceae* family and high-efficient transgene
expression can be easily obtained in *Nicotiana benthamiana,* this tobacco species was
used in the following experiments.

Compared to viral vectors, the *Agrobacterium* system generally won’t lead to
systemic expression of the foreign genes, because the transgene expression is restricted
to the infiltrated areas. For viral RNA, it can systemically spread through
plasmodesmata between cells and via phloem over long distances after inoculated on
leaves (Qi et al., 2004). Considering the similarity of the movement between mobile
RNAs in plant and viral RNA, we hypothesize that selected plant RNAs can also move
from cell-to-cell, or even enter into the vascular tissues and translocate to a distant
organ from the original expression site after agroinfiltration. This system was used
here to study the movement of the following RNAs: full-length StBEL5 (BEL5-FL,
2703 nt), StBEL5 coding sequence only (BEL5-cds, 2076 nt), and StBEL14 (BEL14, 1731 nt).

Previous data showed that StBEL5 RNA was present in the phloem, but not StBEL14, suggesting the mobile ability of StBEL5 RNA. Grafting assays also demonstrate StBEL5 RNA can move from the scion of the transgenic StBEL5 over expression line to the wild type stock (Banerjee et al., 2007). Both the coding sequence and full-length of StBEL5 RNA can move through the graft union. However only the full-length StBEL5 transgenic line can overcome the LD inhibitory effect on tuber formation. And a preferential accumulation of the transgenic StBEL5-FL RNA in stolons under SD was observed, but not for transgenic StBEL5-CDS RNA (Banerjee et al., 2007). All these data indicate UTR parts of StBEL5 RNA may facilitate its movement to the stolons which result in the enhanced tuber formation. A movement comparison was made of the different transcripts of StBELs in this study using the agroinfiltration method.

**Materials and methods**

*Plant materials*

Tobacco (*Nicotiana benthamiana*) plants were grown in a growth chamber maintained at 22 °C under 12 h light for 7 weeks until they reached the 10 leaf stage.

*Plasmid constructs*

The full-length sequence of StBEL14 (Access. # AF406700), the coding sequence of StBEL5 (Access. # AF406697) and full-length sequence of StBEL5 including 5' and 3'UTR sequences, were cloned into the binary vector pCB201 (Xiang
et al., 1999) driven by the Cauliflower Mosaic Virus 35S promoter (CaMV-35S) with a nos terminator. The cassettes were then transferred to Agrobacterium strain GV2260 (Chen et al., 2003; Banerjee et al., 2006). For vector construction of GAS:BEL5, GAS:BEL14, the CmGAS promoter of 1.8 kb was PCR amplified using plasmid pSG3K101 (Ayre et al., 2003) as template and primer pair 5’Xba I GCTCTAGATGACTTGGATTAATTCTCTAAC and 3’SmaI AACCCGGGATTGACTTTGGTGCTTT and cloned in pBI101.2. Subsequently, the reporter β-glucuronidase (GUS) gene present in the vector was replaced by full length StBEL5 or StBEL14 (by Chatterjee) (Figure 4).

pBI121 plasmid which contains the CaMV-35S:GUS and the modified pBI101 which contains GAS:GUS were used as the control, and transferred to Agrobacterium strain GV2260 as described before.

Agroinfiltration

Individual Agrobacterium colonies were grown for 20 h in 5 ml cultures (YEP media plus 50 μg/ml kanamycin) at 30 °C. This was used to inoculate a 50-ml culture (YEP media, 50 μg/ml kanamycin), which was grown for 5 h at 30 °C until OD 600 reading reached 0.5. The bacteria were harvested by centrifuging at 3000 g for 15 min at room temperature, resuspended in infiltration medium (10 mM MgCl2 /10 mM MES, pH 5.7/150 μM acetosyringone). Bacterial densities were adjusted to OD600=0.5, and incubated at room temperature for a minimum of 3 h. 1 ml syringe (without a needle) was used, the tip of the syringe was pressed against the underside of the attached leaf lamina while simultaneously applying gentle counterpressure to the other
side of the leaf. The Agrobacterium solution was injected into the airspaces inside the leaf through the stomata, or through a tiny incision made to the underside of the leaf.

For movement assays, zones at the tip of tobacco leaves were injected with the mixtures of P19 transformed Agrobacterium culture and specific StBEL or empty vector transformed culture. P19 was added to inhibit the post-transcriptional gene silencing (PTGS). Leaves, petioles and stems were harvested 4 day post injection (4dpi) and the RNA was extracted from these sample tissues and used as the template in one round of RT-PCR reactions.

**GUS assay**

Leaves were evaluated histochemically for GUS activity after agroinfiltration following Jefferson et al. (1987).

**RNA isolation and RT-PCR**

Total RNA was isolated from collected Nicotiana benthamiana tissue samples by using the RNeasy Plant Mini Kit (Qiagen). 20 ng total RNA was used in the following RT-PCR. Gene specific primers for StBEL5 and -14 were used with the transgene-specific primer, NT-2 (NT-2 primer was applied to discriminate transgenic RNA from native BEL5 homologues).

- NT-2 primer: 5' GCGGGACTCTAATCATAAAAAC 3';
- StBEL5-(CBEL5F): 5'GAATTCTTCACCAGCAGCAGC3';
- StBEL14-F: 5' ACAACATGGTAGAGTG 3'

18S Ribosomal RNA was used as an internal control. Primers for 18S rRNA were provided in the manufacturer’s kit (Ambion QuantumRNA universal 18S, cat # 1718).
VirA-F: 5'AGGCAGTTGGTACACTTG3' / VirA-R: 5'GCGGCGATCTTGTCTTC3'

The RT-PCR for StBEL RNAs was set to the following condition (50 °C for 30 min, 94 °C for 2 min, 94 °C for 30 s, 54 °C for 30 s, 68 °C for 30 s, followed by 1 cycle of incubation at 68 °C for 5 min). After determining the linear range for both PCR products, 20 PCR cycles were used for the 18S rRNA and 34 cycles for amplification of the StBEL products.

Results

As a rapid assay, agroinfiltration system was used to assess the mobility of several potato BEL RNAs. DNA templates for test RNAs were cloned into two binary vectors with different promoters. A CaMV-35S promoter drove expression for all three constructs in the binary vector, pCB201 (Figure 4). The CaMV-35S promoter is a strong constitutive promoter (Odell et al., 1988), exhibits a high level of transcriptional activity in a variety of plant tissues (Williamson et al., 1989) and confers a high level of expression within most cells when transferred into plants (Benfey and Chua 1990; Schnurr and Guerra 2000). To exclude the possibility of artefactual movement caused by the strong 35S promoter, DNA templates for test RNAs were also inserted into the modified pBI101 binary vector which contains a GAS promoter and a nos terminator (Figure 4). This promoter originates from Cucumis melo leaf-specific galactinol synthase promoter. Its activity is detected only in the companion cells of leaf minor veins (Ayre et al., 2003; Haritatos et al., 2000).

Vectors were transformed into Agrobacterium and infiltrated into the abaxial side of the terminal portion of intact leaves from Nicotiana benthamiana plants. Leaf
structure and physiological condition affected infiltration greatly. Fully extended old leaves have low efficiency, while partially extended young leaves have great variation among different leaves. The 7-week old plants with fully extended leaves show the high efficiency and repeatability in agroinfiltration assay. Leaves in the upper middle parts of the plant were chosen (Figure 5). *Agrobacterium* suspension can penetrate into the *Nicotiana benthamiana* tissue easily and circular infiltrated regions will appear after a successful infiltration.

To assess the effectiveness of the system and the efficiency of expression, parallel experiments were performed with constructs pBI121 and modified pBI101 plasmids containing the GUS marker. We examined the gene expression level as monitored by GUS (Jefferson *et al.*, 1987). The maximum GUS staining was observed 4 dpi. Intensive GUS staining was observed when its expression was driven by 35S promoter (Figure 6B), and only tiny amount of GUS staining was observed in the minor veins of the leaf when its expression was under the control of GAS promoter (Figure 6A).

Therefore, sample tissues were collected 4 dpi from the infiltration site (Figure 5, L), the petiole (Figure 5, P) and the lower portion of the stem (Figure 5, S). Three RNAs were tested: full-length *StBEL5* RNA (Figure 4, p35S:BEL5-FL, pGAS:BEL5-FL 2730 nt), *StBEL5* coding sequence only (Figure 4, p35S:BEL5-cds, 2076 nt), and *StBEL14* (Figure 4, p35S:BEL14, pGAS:BEL14, 1731 nt). NT-2 primer was applied to discriminate transgenic RNA from native *BEL5* homologues. Based on the PCR product, the amount of selected RNA in stems of infiltrated plants was greatest
with the *StBEL5-FL* construct, followed by the *StBEL5-cds* and *StBEL14* constructs (Figure 7A, left panel). Ribosomal RNA was used as an internal control (Figure 7A, right panel). Similar results were obtained when the pGAS:BEL5-FL transformant was co-infiltrated with pGAS:BEL14 transformant (Figure 7B). Identity of PCR products was confirmed by sequencing the isolated bands. These results demonstrated a clear difference between the degree of mobility for *StBEL5* and *StBEL14* transcripts in this model system (Figure 7A, B).

To determine if the *Agrobacterium* cells would move long distance, *Agrobacterium* specific gene *VirA* was tested. *VirA* gene is encoded in the Vir region on the *Ti* plasmid. This region contains 6-9 transcriptional units (virA, B, C, D, E, F, G, H, J) (Gelvin, 2006). Some *vir* gene products act within the bacterial cell while others are transported to the host cell. VirA is associated with the bacterial inner membrane, in a complex with chromosomal gene product ChvE. Binding of an appropriate phenolic inducer molecule to the VirA complex causes activation of its latent protein kinase activity. Transcripts of *VirA* gene were detected at the *Agrobacterium* injection site, not the distal part such as petiole or stem (Figure 7B). These results confirm that the observed transcripts in petiole and stem was due to the RNA movement.

**Discussion**

Agroinfiltration is a popular transient gene expression method, and it is often used as a delivery system for replicons that either move systematically or amplify locally (Marillonnet *et al.*, 2005). This system was used in this long-distance mobile RNA study. Compared to the classical heterografting experiment, agroinfiltration is
much easier to handle, and can at least provide some indications of whether certain transcripts can move and how well they move without performing grafting experiments.

However, several critical concerns should be taken when applying agroinfiltration in mobile RNA study. To avoid false negative results due to the low transformation efficiency at the injection site, acetosyringone was included in the suspension buffer. It helps *Agrobacterium* cells attach to the plant cells and also activates the virulence genes on the Ti plasmid during the infection process.

Post-transcriptional gene silencing (PTGS) is another limiting factor in *Agrobacterium*-mediated transient gene expression especially in *Nicotiana benthamiana*. To enhance transient gene expression, p19 (from tomato bushy stunt virus, TBSV) the viral silencing suppressor was co-expressed with our constructs. *Agrobacterium* cultures of pCB201:BELs and 35S:p19 were mixed in a 1:1 ratio (Voinnet *et al.*, 2003). In this study, high levels of gene expression were achieved at the injection sites.

From the preliminary agroinfiltration data, *StBEL5-FL* RNA shows the most robust mobile ability comparing to *StBEL5-CDS* RNA or *StBEL14* RNA. These results are consistent with the data from Banerjee (2007). The UTR portions of *StBEL5* RNA facilitate its long-distance movement or enhance its RNA stability.

*StBEL14* RNA was absent in the potato phloem cells indicating it may not have long-distance mobile capacity. Here, the amount of *StBEL14* RNA in the distal stem part is the least compared to that of *StBEL5*. Movement of GUS RNA was also
observed using agroinfiltration (data not shown). The low level of unselected RNA movement appears to be a shortcoming of this assay. The inclusion of appropriate controls is critical in utilizing this assay to monitor RNA movement.
CHAPTER 4. A FUNCTIONAL STUDY OF STBEL5 UTRS

Introduction

The long-distant movement of \textit{StBEL5} RNA was confirmed by heterografting experiments. Transgenic \textit{StBEL5} coding sequence (cds) over-expressing lines (\textit{StBEL5}-CDS: a truncated form of the \textit{StBEL5} RNA, without neither 5' UTR nor 3' UTR) and \textit{StBEL5} full length over-expression lines (\textit{StBEL5}-FL: this construct included 146 nt of 5' UTR and the complete 505 nt of 3' UTR) were grafted onto wild type stocks respectively. In both cases, transgenic \textit{StBEL5} RNAs were detected at the wild type stolon tips suggesting \textit{StBEL5} RNA can go through the graft-union no matter whether it has UTR parts or not (Banerjee \textit{et al.}, 2007).

Both of the transgenic lines produced more tubers at a faster rate than wild type plants under SD conditions (Chen \textit{et al.}, 2003; Banerjee \textit{et al.}, 2006). However \textit{StBEL5}-FL transgenic lines present a unique feature, tubers were produced from these lines grown in soil even under LD conditions, a very strict non-inductive condition for tuber formation. While no tuber formation was observed for either wild type plants or \textit{StBEL5}-CDS over expression lines under such conditions (Chen \textit{et al.}, 2003). Addition of both the 3' and 5' UTRs in over-expression lines of \textit{StBEL5} clearly affected the timing and extent of tuber formation. The \textit{StBEL5}-FL transgenic lines exhibited increased earliness, tuber numbers and overall yields. The addition of the UTRs in transgenic constructs confers soil-grown plants the ability to overcome LD inhibitory effect on tuber formation. Further study revealed a preferential accumulation of the
transgenic \textit{StBEL5-FL} RNA in stolons under SD, but not for transgenic \textit{StBEL5-CDS} RNA (Banerjee \textit{et al.}, 2007).

All these data indicate UTR parts of \textit{StBEL5} RNA contribute to the observed SD facilitated tuber formation. In eukaryotic cells, both 5′ UTR and 3′ UTR are involved in the post-transcriptional regulation of gene expression processes, including translation efficiency control, modulating mRNA subcellular localization and mRNA stability regulation (Le and Maizel, 1997; Oleynikov and Singer, 1998; Pesole \textit{et al.}, 2001; Mignone \textit{et al.}, 2002; Chabanon \textit{et al.}, 2004). These processes are mainly controlled by cis-acting functional elements in the UTRs, which comprise both sequence motifs and RNA structure motifs. These motifs may be potential binding sites for chaperone proteins. With 505 nt, \textit{StBEL5} RNA has the longest 3′ UTR among the 7 known potato BEL RNAs. While \textit{StBEL14}, the only known BEL RNA that was not detected in phloem cells has a 3′ UTR of only 90 nt. Considering the mean length of plant 3′ UTR is around 200 nt (Mazumder \textit{et al.}, 2003), the greater length of \textit{StBEL5} 3′ UTR may imply its multiple regulation roles.

First we speculated that the UTRs of \textit{StBEL5} could control its expression. The extra UTR sequence may improve the efficiency of translation, making more of the protein available to bind with putative target genes and enhance the tuberization response. There are numerous reports of identification of RNA sequences that mediate enhanced protein synthesis. (Gualerzi \textit{et al.}, 2003; Arroyo-Helguera \textit{et al.}, 2005; Barreau \textit{et al.}, 2006; Ortega \textit{et al.}, 2006). A wheat germ \textit{in vitro} translation system was used to test the function of extra UTR sequences in translation control. Four different
StBEL5 transcripts were tested: the StBEL5 Full length RNA (StBEL5-FL), the coding sequence region of StBEL5 RNA (StBEL5-CDS), 5’UTR truncated fragment of StBEL5 RNA (StBEL5A) and 3’UTR truncated fragment of StBEL5 RNA (StBEL5B) (Figure 8).

The UTRs of StBEL5 may be involved in the preferential movement of the StBEL5 transcript through phloem to the stolon tip under SD. This targeted movement would increase the concentration of the transcript in stolon tips, leading to increased translation and subsequent enhanced activity by StBEL5 transcription factor in coordination with other partners. Recently more selected long-distance transport of endogenous transcripts has been reported (see review, Lough and Lucas, 2006), but the underlying machinery is not understood.

In other systems, specific motifs in RNA sequences designated as “localization elements” (LE) or “zip codes” have been identified, and have been regarded as a common mechanism for intracellular localizing RNAs (Lewis et al., 2004). These elements may be located anywhere in the transcripts, usually in 3’UTR. In some cases the interaction of the two separated elements is required for the correct localization (Corral-Debrinski et al., 2000; Thio et al., 2000, Lewis et al., 2004). In addition to primary sequence, the information provided by the secondary structure within the LE is also important for the recognition of the trans-acting factors that direct localization.

Such localization motifs have been reported in potato spindle tuber viroid RNA characterized as a bipartite RNA sequence that facilitates selective movement
through plant cells (Qi et al., 2004). Other plant viral RNAs are found to traffic as ribonucleoprotein complexes (RNPs) with movement protein (MP) or the virus replication complex (Ding, et al., 1995; Fujiwara, et al., 1993). Less is known about localization elements in plant endogenous RNAs, even though many long-distance mobile transcripts in plants have been confirmed by heterografting experiments. In order to have a better understanding of how these mobile RNAs are recognized and translocated, identification of RNA binding proteins is critical. Maize homeodomain protein KNOTTED1 (KN1) was the first plant endogenous protein shown to traffic its RNA from cell to cell (Lucas et al., 1995). CmPP16, a Cucurbita maxima phloem protein mediates its RNA movement between companion cells and sieve elements (Xoconostle-Cázares et al., 1999). In this work, EMSA and northwestern blots were applied for identifying StBEL5 RNA binding proteins in potato.

**Materials and methods**

*Plasmid constructs*

Four different StBEL5 transcripts were PCR generated using plasmid pCB201-StBEL5 (Banerjee, 2006) as template. StBEL5-FL was amplified using primer pair: 5' CTCTAGAATCAGTCTGACAAGAAGGCAA 3'

5' CGAGCTCTGCAATTACAAGCAATGGAAAC 3'

StBEL5-CDS: 5' CAATCTAGACAGATATGTACTATCAAGGAA 3'

5' CATGAGCTCCAAAATCTGGTAATAGTTGAGT 3'

StBEL5A: 5'CTCTAGAATCAGTCTGACAAGAAGGCAA 3'

5' CATGAGCTCCAAAATCTGGTAATAGTTGAGT 3'
StBEL5B: 5’ CAATCTAGACAGATATGTACTATCAAGGAA 3’
5’ CGAGCTCTGCAATTACAAGCAATGGAAAC 3’

These PCR fragments were digested with Xba I and Sac I and cloned into Xba I–Sac I-digested pGEM-4Z with T7 promoter.

In vitro transcription and in vitro translation

All RNAs were transcribed in vitro from Sac I-linearized plasmids using the T7 Megascript kits for uncapped RNA and T7 Mmessage machine kits for capped RNA (Ambion). 0.2 pmol RNA transcript was added to the wheat germ extract (Promega) in a final reaction volume of 12.5 µL and translated for 2 h at room temperature. For radiolabeling of in vitro translated products, 10 µCi of 35S-labeled methionine was added to the translation reaction. Total protein from the translation mix was separated on a precast 4%–12% polyacrylamide gel (Invitrogen). Gel was fixed in the fixing solution (50% methanol, 10% glacial acetic acid, 40% water) for 30 min, then soaked in 7% acetic acid, 7% methanol and 1% glycerol for 5 min to prevent cracking during drying. Gel was then dried, exposed to a PhosphorImager screen for 24 h, and scanned on a STORM 840 PhosphorImager (Molecular Dynamics).

Results

In vitro translation assay was used to compare the translation efficiency of the four different transcripts: StBEL5-FL, StBEL5-CDS, StBEL5A and StBEL5B (Figure 8). No difference was observed (Figure 9A). Considering most eukaryotic mRNA have m7GpppN cap at the 5’ end, which is important for the binding of translation initiation factors and contributes to mRNA stability, capped RNA templates for in vitro
translation assay were also prepared using mMESSAGE mMACHINE kit. Only a small amount of translation enhancement was achieved, but still no difference among the four different transcripts (Figure 9B). In wheat germ extract system, many factors affect translation efficiency of specific RNAs. In this study, the optimal mRNA concentration was determined at 0.4 pmol and optimal K⁺ concentration was set at 40 mM for a final reaction volume of 12.5 μL (Figure 10).

The expected translation product size is around 76 kDa. Unexpectedly, three major bands were observed in that region. It is possible that some cleavages may have occurred in the *in vitro* translated peptide.

EMSA and northwestern assay were used to assess a possible interaction of *StBEL5* RNA and its binding proteins from potato leaves and petioles, however no protein from these samples was detected specifically binding to the *StBEL5* RNA yet.

**Discussion**

Previous work demonstrated that *StBEL5* mRNA was presented in potato phloem cells, SD photoperiod can mediate its movement to the target organ, and transgenic lines that over-express the full-length *StBEL5* transcript have the capacity of overcoming the LD inhibitory effect on tuberization (Banerjee *et al.*, 2007).

Both the unique movement pattern of full-length *StBEL5* transcript in agroinfiltration experiment and its LD tuberization promoting ability compared to the truncated *StBEL5* transcripts indicate the important role of *StBEL5* UTR parts.

In this study, *in vitro* translation system was used to test whether extra UTR sequence in StBEL5 functions in enhancing the translation efficiency. No such
enhancement was observed. Considering most eukaryotic mRNAs have the m7GpppN cap at the 5’ end, capped StBEL5 transcripts were prepared. However, no enhanced translation occurred, whatever the RNA templates are capped or not. It has been reported in wheat germ extract, many transcripts do not require a cap structure for efficient translation. Besides the template context, there are many other factors which would affect translation efficiency of specific RNAs in wheat germ extract system. The concentration of both the mRNA template and the potassium were optimized, still no difference of translation efficiency was found among the four StBEL5 transcripts. The three bands of the final translation products were thought to be the results from protein cleavage, since there’s only one large sized ORF existing in all the StBEL5 RNA templates. From this wheat germ extract system, StBEL5 full length transcripts didn’t have the enhanced translation ability comparing to the UTR truncated transcripts. In some cases, UTR are not necessary for translation, even one nucleotide in addition to the coding sequence can trigger translation. Further in vivo translation assay may provide information about whether the observed result was specific for the in vitro translation system or not.

Alternatively, UTRs of StBEL5 may facilitate its preferential movement through phloem to the stolon tip under SD. RNA trafficking was first found in the study of viral infection, during which viral RNA genomes move as RNP complexes with viral movement proteins (MPs) (Ding et al., 1995; Fujiwara, et al., 1993). MPs are known to interact with plasmadesmata (PD) to increase their size exclusion limit (SEL), to traffic themselves and to facilitate spread of the virus (Waigmann et al., 1994). Recently a
A bipartite RNA sequence motif was revealed to direct potato spindle tuber viroid trafficking from bundle sheath cells to mesophyll cells (Qi et al., 2004). The systemic viral RNA or viroid movement in phloem is thought to mimic the long-distance mobile signals in plant. But in contrast to the free movement of viral RNP, the translocation of selected endogenous transcripts or proteins in higher plants is selective. For example, the movement of knox genes in Arabidopsis is selective; KNAT2, -3, -4, and -6 did not move, but KNAT1 can (Hake et al., 2004). This selective functional long-distance movement has also been reported for several other plant endogenous transcripts.

PFP-LeT6, the mutant transcripts of tomato KN1-related homolog gene can traffic from a Mouse ears (Me) stock into a wild-type graft scion and induce Me morphology in the scion shoot. The GAI RNA moves through the phloem to the shoot apex in Arabidopsis, tomato and pumpkin, and involves in the leaf development regulation (Haywood et al., 2005). However, it is unclear how these transcripts traffic to the target site through the phloem.

Other than RNAs, many long-distance mobile phloem proteins have also been demonstrated by heterografting experiments (Golecki et al., 1999; Ishiwatari et al., 1998; Schobert et al., 2000; Thompson and Schulz, 1999). Some of the mobile proteins can bind RNAs and exhibit the destination selective movement of these RNP complexes (Aoki et al., 2005). CmPP16, an MP related Cucurbita maxima phloem protein mediates its RNA movement between companion cells and sieve element (Xoconostle-Cazares et al., 1999); CsPP2, the cucumber phloem protein 2 interacts with Hop Stunt viroid RNA and assist the long-distance movement of the viroid RNA.
(Gómez and Pallás, 2004). Several other melon phloem proteins, CmmPP2 and CmmLec17 were also found binding to certain viroid RNAs (Gómez et al., 2005). Such RNA–protein interactions may contribute to the recognition and transport of a RNA to its selective destination. Other bound factors to this RNP complex may also facilitate its selective movement and differentiate it from many other non-localized RNAs.

The so-called “localization elements” (LE), the designated specific motifs in RNA sequences have been regarded as a common mechanism for the recognition of localized RNA and its bind proteins. These LEs have been identified in mRNAs from other system too, for example, the bicoid (MacDonald and Kerr, 1997, 1998), oskar (Kim-Ha et al., 1993), nanos (Gavis et al., 1996) mRNAs in Drosophila, actin mRNA in fibroblasts (Kislauskis et al., 1994), and VegT (Bubunenko et al., 2002; Kwon et al., 2002), and Vg1 (Deshler et al., 1997; Gautreau et al., 1997) mRNAs in Xenopus. These LE may locate anywhere in the transcript, and in some cases the interaction of the two separated elements is required for the correct localization (Corral-Debrinski et al., 2000; Thio et al., 2000, Lewis et al., 2004). It has also been mentioned that both a start codon and specific 3’ regulatory sequence are required for the intracellular distribution of specific RNAs (Choi et al., 2000).

It is not known yet whether such localization element also exists in UTRs of StBEL5 RNA. The identification of specific StBEL5 RNA binding protein in further research may provide pivotal cues to understand specific RNA-protein interaction in this long-distance signaling pathway.
Figure 1. Transverse section of potato stems using different fixatives: paraformaldehyde (A), ethanol-acetic acid (3:1) (B), 100% acetone (C). Scale bar: 50 μm.
Figure 2. 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). (Xy: xylem; Ph: phleom; Ep: epidermis)
Figure 3. Identification of specific RNAs in potato stem phloem cells by LMPC/RT-PCR. A: Selected cells from xylem (X), phloem (P) or epidermis plus some collenchyma cells (E) were excised by focused laser light and catapulted into the collection microfuge tube cap.

B: Total RNA was extracted from LMPC collected cells and used as the RT-PCR template for detection tissue specific transcripts. StBEL5 RNA was present in xylem, phloem, and epidermal cells. StBEL14 RNA was not detected in phloem cells. NT is nitrate transporter gene, specific for root cells, used as the negative control. G2 is the G2-like transcription factor gene, specific for phloem cells.

C: Total RNA extracted from phloem cells was used as RT-PCR template for detection phloem localized transcripts. Except StBEL14 RNA (lane 5), all the other StBELs RNA (2: StBEL5, 3: StBEL11, 4: StBEL13, 6: StBEL22, 7 and 8: StBEL29, 9: StBEL30) and POTH1 RNA (lane 1) were detected by the RT-PCR.
Figure 4. Constructs of *StBEL5* full-length sequence, coding sequence and *StBEL14* into the binary vector pCB201 with 35S promoter (p35S:BEL5-CDS, p35S:BEL5-FL, p35S:BEL14) and the modified pBI101 with GAS promoter (pGAS:BEL14, pGAS:BEL5-FL).

35S: Cauliflower Mosaic Virus 35S promoter
GAS-pro: galactinol synthase promoter
5′UTR: 5′ untranslated region; 3′UTR: 3′ untranslated region
BEL5cds: coding sequence region of *StBEL5*
BEL14cds: coding sequence region of *StBEL14*
NOS-pro: nopaline synthase promoter
NOS-ter: a terminator sequence isolated from the 3′ end of the nopaline synthase gene
NPTII: selectable kanamycin resistance gene
LB: left border sequence of T-DNA
RB: right border sequence of T-DNA
Figure 5. Four days after infiltration, sample tissues were selected from the site of infiltration (L), the petiole (P), and the lower portion of the stem (S).
Figure 6. Expression of GUS gene in *Nicotiana benthamiana* leaves at 4dpi. pBI121 plasmid which contains the p35S:GUS and the modified pBI101 which contains pGAS:GUS were transferred to *Agrobacterium* strain GV2260 separately. The transformed *Agrobacterium* was mixed at 1:1 ratio with the *Agrobacterium* culture containing viral silencing suppressor p19. GUS expression assays were carried out 4 days after agroinfiltration. A: tiny amount of GUS staining was observed in the minor veins of the leaf when its expression was under the control of GAS promoter (pGAS:GUS); B: Intensive GUS staining was observed when its expression was driven by 35S promoter (p35S:GUS).
Figure 7. RT-PCR product of RNA expressed from collected samples in agroinfiltration assay. A: Constructs of StBEL5-cds, StBEL5-FL, and StBEL14 in pCB201. Infiltration was performed for each of the cultures separately with 35S:p19 Agrobacterium culture mixed in at 1:1 ratio. C: constructs of StBEL5-FL, and StBEL14 in modified pBI101 vector. Total RNA was extracted from samples collected 4dpi from the injection site (L), as the positive control site; the petiole (P), and a portion of the stem below the node (S). The DNA products generated are from a one-step RT-PCR reaction. Equal volumes of the PCR reactions were loaded for each set (L, P, S). C: 18s ribosomal RNA primers were used to generate an internal control. Agrobacterium specific gene VirA was used as the negative control.
Figure 8. Different fragments of StBEL5 RNA were transcribed *in vitro* under T7 promoter.

StBEL5-FL: this construct included 146 nt of 5'UTR and the complete 505 nt of 3'UTR and the coding sequence.

StBEL5-CDS: a truncated form of the *StBEL5* RNA, without neither 5'UTR nor 3'UTR, only contains the coding sequence.

StBEL5A: 5'UTR truncated form of the *StBEL5* RNA.

StBEL5B: 3'UTR truncated form of the *StBEL5* RNA.
Figure 9. In vitro translation of the four transcripts: StBEL5-FL (FL, full length StBEL5 RNA), StBEL5-CDS (CDS, coding sequence of StBEL5 RNA), StBEL5A (5A, 5'UTR truncated transcript) and StBEL5B (5B, 3'UTR truncated transcript). 0.2 pmol each transcript was added to the wheat germ extract in a final reaction volume of 12.5 μl. After 2 h reaction, all the translated proteins were loaded on a 4%-12% polyacrylamide gel. BMV: Brome Mosaic Virus RNA is the positive translation control. (A. uncapped RNA templates; B. Capped RNA templates)
Figure 10. Optimization of the potassium concentration and RNA template concentration in translation. A gradient of final potassium concentration was tested at 40 mM, 80 mM, 120mM and 160 mM. And the StBEL5-FL RNA was diluted from 0.4 pmol to 0.2 pmole and 0.1 pmol. The optimal potassium concentration was set at 40 mM and 0.4 pmol RNA was used in translation of the StBEL5-FL RNA.
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