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ORIGINAL ARTICLE

Accumulation of *BEL1*-like transcripts in solanaceous species

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Abstract Although numerous RNAs have been detected in the phloem, only a few have been confirmed to move long distances. In potato, full-length mRNA of the *BEL1*-like transcription factor, StBEL5, moves from leaf veins through the phloem to stolon tips to activate tuber formation. BEL1-like transcription factors are ubiquitous in plants and interact with KNOTTED1-types to regulate numerous developmental processes. To explore the range of *KNOTTED1*- and *BEL1*-like mRNAs present in phloem, an analysis of the transcript profile of phloem sap was undertaken. Using a modified technique for the collection of phloem-enriched exudate from excised stems, numerous RNAs encoding these transcription factors were detected in the phloem sap from several solanceous species. All seven known *BEL1*-like RNAs of potato were detected in the phloem-enriched exudates of stem, whereas several stolon-abundant RNAs were not. After refining the technique to minimize the contamination from RNA arising from wounded cells, *KNOTTED1*-like RNAs were detected in phloem-enriched sap of potato and *BEL5* RNA was detected in the sap collected from two closely related non-tuber-bearing potato species and tomato. *BEL5* RNA was also detected in RNA extracted from leaf veins of tobacco. The detection of these full-length mRNAs from the *KNOTTED1*- and *BEL1*-like families in phloem sap indicates that their potential role as long-distance signals seems to be much more extensive than previously known.

Keywords Tuberization · Mobile RNA · TALE superfamily · Potato

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Abbreviations
UTR Untranslated regions
TALE Three amino acid loop extesnion

Introduction

As a part of an elaborate long-distance communication system, plants have evolved a unique signaling pathway that takes advantage of connections in the vascular tissue, predominately the phloem. This information pathway has been implicated in regulating development, responding to biotic stress, delivering nutrients, and as a vehicle commandeered by viruses for spreading infections (Lough and Lucas 2006). Co-suppression mediated by systemic-acquired gene silencing and the transport of miRNAs also involves movement through the phloem (Sonoda and Nishiguchi 2000; Crete et al. 2001; Brosnan et al. 2007; Buhtz et al. 2008). Recently, the transport of full-length mRNA through the phloem has been identified as a key component of long-distance signaling (reviewed by Kehr and Buhtz 2008).

Whereas hundreds of RNAs have been identified in phloem (Asano et al. 2002; Vilaine et al. 2003; Doering-Saad et al. 2006; Omid et al. 2007), only six have been confirmed via grafting experiments to be transported (Kehr and Buhtz 2008). Of these, *CmGAIP*, *CmNACP*, and *CmPP16* are from *Cucurbita maxima* (Haywood et al. 2005; Ruiz-Medrano et al. 1999; Xoconostle-Cazares et al. 1999), *DELLA-GAI* is from *Arabidopsis* (Haywood et al. 2005), *PFP-LeT6* is from tomato (Kim et al. 2001), and *StBEL5* is from potato (Banerjee et al. 2006). Using the *Cucurbita* species, scions of cucumber grafted onto pumpkin stocks provided direct evidence that specific pumpkin mRNAs were translocated through the heterograft via the phloem

into the shoot apex (Ruiz-Medrano et al. 1999; Xoconostle-Cazares et al. 1999). The discovery of the RNA-binding protein, CmPP16, provided additional support for the long-distance transport of RNA in pumpkin (Xoconostle-Cazares et al. 1999). Of this group of six RNAs, however, information on the dynamics of movement is available only for *StBEL5* RNA (Banerjee et al. 2006).

StBEL5 is a member of the TALE superfamily of transcription factors (Bürglin 1997). The BEL1-like family of transcription factors is ubiquitous among plant species and interact with KNOTTED1-types for targeting genes to regulate numerous developmental processes (Bellaoui et al. 2001; Müller et al. 2001; Smith et al. 2002; Smith and Hake 2003; Bhatt et al. 2004; Kanrar et al. 2006). In potato, the BEL1 transcription factor, StBEL5 and its Knox protein partner, POTH1, regulate tuber formation by mediating hormone levels in the stolon tip (Rosin et al. 2003; Chen et al. 2003; 2004).

RNA detection methods and heterografting experiments demonstrate that *StBEL5* transcripts are present in phloem cells and move across a graft union to localize in stolon tips, the site of tuber induction (Banerjee et al. 2006). This movement of RNA to stolon tips is facilitated by a short-day photoperiod, mediated by sequence tags present in the untranslated regions of the *StBEL5* transcript, and correlated with enhanced tuber production (Banerjee et al. 2006). Based on these results, the mRNA of *StBEL5* appears to act as a mobile signal that is delivered to the stolon tip to induce tuber formation. Tuberization is a specialized developmental process, yet sequence motifs present in the 3′ UTR of *StBEL5* are conserved in the *BEL5* mRNAs of both tomato and tobacco. Are BEL5 RNAs present in the phloem of plant species that do not make tubers? Are other transcripts from the TALE superfamily of transcription factors present in phloem? The results of this study confirm the presence of several *BEL1*- and *KNOTTED1*-like RNAs in the phloem sap of potato and other nontuber-bearing solanaceous species suggesting that movement of these full-length mRNAs plays a much wider role in long-distance signaling than previously assumed.

Materials and methods

Plant materials

Solanum tuberosum ssp. *andigena* plants were generated from in vitro-grown plantlets established from tuber sprouts. All plantlets were grown on a media containing 2% sucrose plus MS basal salts (Murashige and Skoog 1962) under long-day conditions (16 h light, 8 h dark) at 21°C. Plants of *Solanum etuberosum* (PI 498311) and *S. palustre* (PI 558259) were generated from the seeds obtained from the Potato Introduction Station (Sturgeon Bay, WI, USA). Both of these Andean potato species do not form tubers. Rooted cuttings from healthy stock plants of *S. etuberosum* and *S. palustre* were transferred to growth chambers under long-day conditions for 2 weeks before starting any experiments. *Nicotiana tabacum* var. Petit Havana and *S. lycopersicum* plants were grown from seed and transferred to 10-cm pots. All plants were grown under long-day conditions (16 h light, 8 h dark) either in the greenhouse or in a growth chamber at approximately 21°C. Sap was collected from stock plants after they were grown for approximately 3 weeks.

RNA extraction, sap collection and RT-PCR

Total RNA was extracted from leaf, stem, and root samples according to the manufacturer's instructions (RNeasy® Plant Mini Kit, Qiagen). Samples were incubated for 1 min at room temperature before eluting the column. RNA samples were treated with a RNase-free DNase (TURBO DNA free™ kit, Ambion) before PCR.

Two hours prior to collection of sap, source plants were thoroughly watered. Immediately, prior to harvest, plants were placed in a tray with excess water. Complete transverse cuts were made across the stem approximately 3.0 cm above the soil level with a clean razor blade. The stem exudate was blotted with a ChemWipe tissue for 5 min to minimize RNA contamination from disrupted stem cells and sap was collected for up to 30 min (Fig. 4). For harvest (Fig. 4), at least 200 µl of sap was collected across the stem with a 0.2 ml Pipetman and stored on ice. For analysis (Fig. 5), 200 µl of sap was collected during each time interval: immediately upon bleeding, 10–20 min from the onset of bleeding, 20–30 min, and after 30 min from the onset of stem bleeding, all from the same set of plants. Immediately after collection, 500 µl of Trizol reagent was added. The sample was vortexed for 30 s, 0.2 ml chloroform was added, and the sample was again vortexed for 30 s. The sample was then centrifuged (12 K rcf) for 15 min at 4°C. The aqueous phase was removed, placed in a separate tube, and then subjected to ethanol precipitation at −20°C overnight. After washing the pellet with 70% ethanol, the RNA sample was air-dried, resuspended in a minimum volume of nuclease-free water, and quantified using a GeneQuant spectrophotometer (Biochrom, Cambridge, England).

To detect specific mRNAs, sample RNA was reverse-transcribed using SuperScript™ III One Step RT-PCR System with Platinum® Taq DNA Polymerase Kit (Invitrogen, Carlsbad, CA, USA) with 0.25 µM gene-specific primers (Table 1). Primers for *G2*, *NT2*, *POTH1*, *StBEL11*, *−13*, *−14*, *−22*, *−29*, and *−30* were previously described (Yu et al. 2007). All primers were synthesized at the DNA Facility, Iowa State University. The amount of RNA

Table 1 Gene-specific primers used for RT-PCR reactions with expected product size in nucleotides (nt)

Primer	Sequence (5′→3′)	Length of PCR product (nt)
BEL5-1A-F	GCAGATATGTACTATCAAGGAACC	2,742 (Sp)
BEL5-1C-R	CTAGTTGTATCAATCTTCTCAAGG	
BEL14-3A-F	GTGATCATGGTCCTTCGTCTTC	1,606
BEL14-3B-R	TCATGAAGAAGTTGTGCCC	
BEL29-4A2-F	CAAGGGCTTTCACTTAGC	2,028
BEL29-4F-R	CTCCAATGTATAAAGACTGTTACTATG	
BEL30-2A-F	GATGGCGACTTATTTTCCTAGTCC	1,998
BEL30-2D-R	GAAGATGAATGTTGATGTACTCAAACCTTG	
StBEL5-F	GGGAGATTTTGGAAGGTTTG	375
StBEL5-R	TCAAATTGGGTCCTCCGACT	
CEN-F	GATCTGCTTATACACTCGTA	483
CEN-R	GGATCAGTCATGATCTTTTC	
Tup1-F	CATGCAACCCCACTCAGA	398
Tup1-R	CGAGAGCCCACAGCATC	
Gigantea-F	GGCACTATTAACTGGCAAA	435
Gigantea-R	GTCACACTCGCACTGAATA	
NAC-F	CCTTGATGAAGGTCCCCT	335
NAC-R	GCCGTTTTGGTGCGGGA	
RAS-F	CCTCTTTTTCATGGAGACC	294
RAS-R	CCGTATGAACCATGCCAC	
Rubisco-F	GGCAAAGATAAGCACTCAGA	454
Rubisco-R	CCACCTCAGCCAAGACC	
POTH15-F	GCCACGTCAGCAACAAT	361
POTH15-R	CCTGCTTAAGGCTTCCC	
H09cds-F	GGCTCTTCATCAACAACAAC	492
H09cds-R	TGCCGACAGTAGCTGATC	
TomBEL5-F	CGCACTTTTACAGCGTATGT	502
TomBEL5-R	CCTGCTGCTACTTTCACC	
TobBEL5F3	GCCAAGAAATCATGACATG	400
TobBEL5R3	CCTATATGCTAGAAACTGTGTGC	

template used varied among reactions (20–200 ng) due to the estimated abundance level of the target RNA. PCR conditions were 50°C for 30 min; 94°C for 2 min; 38 cycles of 94°C for 30 s, 54–56°C for 30 s, 68°C for 30 s. *G2* RNA (Access # TC118156) was used as a positive control for phloem sap (Zhao et al. 2005) and the root-specific potato RNA (Access # CK267169), homologue to the nitrate transporter (NT) gene of *Arabidopsis* (Nazoa et al. 2003), as a negative control.

Characterization of BEL cDNAs from *Solanum etuberosum* and *S. palustre*

Of the seven known BEL1-family members from *S. tuberosum*, four were chosen for further analysis in the two nontuber-bearing species: *StBEL5, StBEL14, StBEL29*, and *StBEL30*. The first four primer sets listed in Table 1 were used to construct full-length *StBEL5*, *StBEL14, StBEL29*, and *StBEL30,* respectively, from *S. etuberosum* and *S. palustre*. The RT-PCR conditions were similar to those described in the previous section, PCR products were cloned into the TOPO TA vector (Invitrogen, Carlsbad, CA, USA). After selection and plasmid isolation, clones were sequenced at the DNA Facility, Iowa State University. The sequence obtained was screened for matches using the basic local alignment search tool (BLAST). The percent nt matches were determined by a comparison to the known *StBEL* sequences (Table 2).

Wounding experiment

Solanum etuberosum and *S. palustre* stock plants were propagated from the cuttings rooted under mist and placed in a growth chamber under long-day conditions at 21°C until they reached the 10- to 12-leaf stage. Intact plants were then wounded by cutting stems 3.0 cm above the soil

Table 2 A comparison of cDNA clones from the *BEL1*-like family of transcription factors in two nontuber-bearing *Solanum* species

BEL cDNA	cDNA length (nt)	3′ UTR length (nt)	Percent nt match	Protein length (aa)	GenBank access #
Se BEL5	2683	454	94	698	EU686384
Sp BEL5	2742	514	94	698	EU686380
St BEL5	2735	505	–	689	AF406697
Se BEL14	1606	NA	96	511	EU686378
Sp BEL14	1606	NA	96	511	EU686381
St BEL14	1731	130	–	532	AF406700
Se BEL29	2028	321	93	516	EU686385
Sp BEL29	2027	321	93	516	EU686382
St BEL29	2128	423	–	567	AF406702
Se BEL30	1998	59	97	645	EU686379
Sp BEL30	1998	59	97	645	EU686383
St BEL30	2065	57	–	645	AF406703

Se Solanum etuberosum, Sp *Solanum palustre*, St *Solanum tuberosum*, *NA* data not available

The percent nt match is to the *BEL1*-like ortholog in *Solanum tuberosum* (Chen et al. 2003)

level superficially with a clean razor blade or using a hemostat to wound leaf mesophyll several times between major veins. Tissue samples were harvested from three plants per species per time point (0, 24, and 48 h post-wounding), pooled, frozen in liquid nitrogen, and stored at −80°C until RNA extraction.

RT-PCR was performed with 20 ng of total RNA as template and the *BEL5*-specific primers, StBEL5-F and StBEL5-R (Table 1). PCR conditions for *BEL5* quantification were 50°C for 30 min; 94°C for 2 min; 32 cycles of 94°C for 30 s, 56°C for 30 s, 68°C for 30 s. The internal control for PCR reactions was rRNA. PCR conditions for the rRNA were 50°C for 30 min; 94°C for 2 min; 21 cycles of 94°C for 30 s, 56°C for 30 s, 68°C for 30 s. Homogenous PCR products were quantified using ImageJ software (Abramoff et al. 2004) and normalized using the rRNA values. PCRs for *BEL5* and rRNA were standardized and optimized to yield product in the linear range (for example, 32 cycles for the *BEL5* RNA and 21 cycles for the rRNA). Three quantitative RT-PCRs were performed and the standard error calculated.

Results

BEL1-like genes are present in nontuber-bearing *Solanum* species

Using RT-PCR with primers from potato *BEL1*-like genes, several *BEL1*-like cDNAs were identified and sequenced from two very closely related nontuber-bearing solanaceous species, *S. etuberosum and S. palustre* (Table 2). Three of these genes, *BEL5*, *−14*, -and *−30*, were selected as representatives of the major phylogenetic groups of potato (Chen et al. 2003). *BEL29* is closely related to *BEL5* in overall sequence. To compare to existing *BEL1* genes, a phylogenetic analysis of these new genes based on their amino acid sequence was performed (Fig. 1). As expected, the *S. etuberosum and S. palustre* BEL1 proteins aligned very closely to their *tuberosum* (St) and tomato (Sl) counterparts. *Arabidopsis* BEL1 proteins are included in the dendrogram as a reference. These new *BEL1*-like cDNAs exhibited a very high level of nucleotide sequence match with their *S. tuberosum* counterparts (Table 2). The lengths of the 3′ UTRs were also similar as the 505 nt UTR of *StBEL5* was matched by 454-nt and 514-nt 3′ UTRs for *SeBEL5* and *SpBEL5*, respectively. Alignment of the available 3′ UTR revealed a very high level of nt sequence match (Fig. 2), 94 and 93% match for Sp and Se, respectively, for the *StBEL5* UTR (Fig. 2a), a 87% match for both Sp and Se for the *StBEL29* UTR (Fig. 2b), and a 97% match for both Sp and Se for the *StBEL30* UTR (Fig. 2c). As expected, the conserved regions of the BEL1 family, the SKY box, the BELL domain, and the homeodomain, were also conserved in all eight of the new BEL1 proteins examined in this study.

Because of their importance in development (Banerjee et al. 2006), the *BEL5* genes were selected for a more thorough characterization of their expression patterns. Similar to *StBEL5*, the BEL5 RNAs of *S. etuberosum* and *S. palustre* were ubiquitous. Using gene-specific primers and RT-PCR, these RNAs were detected in leaf, stem, and root RNA (Fig. 3a).

Wound induction of BEL5 genes

Previous studies have demonstrated that the promoter of St*BEL5* was activated in response to wounding in stems but

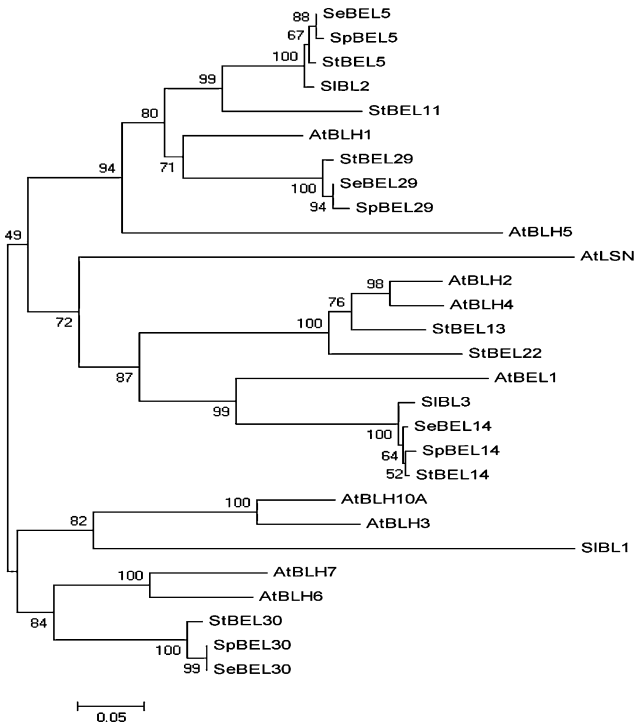

Fig. 1 Phylogenetic analysis of the amino acid sequences of the BEL1-like proteins of potato, two nontuber-bearing *Solanum* species, *Arabidopsis*, and tomato. These data were organized into a phylogenetic tree with the ME-Boot program of the MEGA4 package and the neighbor-joining program (Tamura et al. 2007). The *numbers* listed at the branching points are boot-strapping values that indicate the level of significance (percentage) for the separation of two branches. The length of the branch line indicates the extent of difference according to the scale at the lower left-hand side. *Se Solanum etuberosum*, *Sl Solanum lycopersicum*, *Sp Solanum palustre*, *St Solanum tuberosum*, and *AT Arabidopsis thaliana*

not leaves (Chatterjee et al. 2007). To determine if other solanaceous species exhibited a similar pattern of expression, wound induction for both leaves and stems was examined for *S. etuberosum and S. palustre.* Consistent with the induction pattern of the *StBEL5* promoter, steady-state levels of *BEL5* RNA in both the species were enhanced in wounded stems but not in leaves over 48 h (Fig. 3b, c).

BEL1-like mRNAs are present in phloem-enriched sap

Previous work using in situ hybridization and laser capture microdissection, demonstrated the presence of a several *BEL1*-like RNAs in phloem cells of potato (Banerjee et al. 2006; Yu et al. 2007). These protocols, however, are labor-intensive and time-consuming. To facilitate the analysis of mRNAs in phloem sap, a modified protocol adapted from Buhtz et al. (2008) was implemented. This technique involves collection of sap exudates from excised stems of potato. RT-PCR of RNA extracted from this stem sap revealed the presence of all seven *BEL1*-like mRNAs (Fig. 4a). *G2* mRNA of potato (# TC118156) is a phloem-specific RNA (Zhao et al. 2005) and *NT2* represents a xylem-specific transcript of roots (Nazoa et al. 2003). Several *BEL* RNAs of potato accumulate in stolons during tuber formation (Fig. 4a; Chen et al. 2003). To determine if other stolon RNAs are present in the sap RNA extracted here, several additional RNAs upregulated in stolon tips were assayed (Hannapel 2007). A *Cen1*-like (TIGR access # TC98831), *tup1* (# TC95867), *NAC1* (# TC96473), and *ras*-like (# TC67617) RNA were detected in the stolon tips during the onset of tuber formation but not in the harvested stem sap (Fig. 4b). *Gigantea* (Access # BF154299) was included because of its pivotal role in mediating photoperiod-regulated processes (Sawa et al. 2007). The integrity of the band detected for the *ras*-like transcript in sap RNA (Fig. 4b) could not be confirmed.

Because of the possibility of contamination from the wounded cells of the cut surface of the stem, RNA was extracted from the sap collected at several time intervals after bleeding was initiated. Even though the stem exudates collected in this protocol most certainly contain xylem sap, contamination via this source was not considered a factor because, in a previous study, no RNA was detected in xylem sap (Buhtz et al. 2008). Contamination from the rubisco small subunit RNA (# TC137121) was observed from 0 to 30 min after the initial bleed but not after 30 min (>30, Fig. 5). The two phloem RNAs, *G2* and *StBEL5*, could still be detected even after 30 min of sap collection (Fig. 5). RNA yield from these harvests ranged from 1.4 to 4.2 ng/µl of sap. Based on these results, subsequent analyses of RNA were performed on sap collected after 30 min from the onset of bleeding.

Can other mRNAs be detected in phloem-enriched sap?

Results using the technique for sap collection described in Fig. 4 indicated the presence of all seven *StBEL* RNAs in phloem sap. RT-PCR of RNA extracted from phloem cells harvested using laser capture microdissection detected the presence of a *KNOTTED1*-like mRNA, *POTH1* (Yu et al. 2007). To determine if other *KNOTTED1*-like mRNAs are present in the phloem sap of potato, RT-PCR was performed on total RNA extracted from sap collected at least 30 min after the initial bleed from *S. tuberosum* ssp. *andigena* (Fig. 6a). As expected, *POTH1* RNA was detected in the phloem sap of potato. In addition, *POTH15* RNA, a class-I *KNOX* gene (Tanaka-Ueguchi et al. 1998), and *H09*, a class-II *KNOX* gene (Resier et al. 2000), were also detected (Fig. 6a). Whereas *StBEL14* RNA was not detected previously (Yu et al. 2007), the presence of a low level of its mRNA was observed in RNA from phloem-enriched sap collected in this study (Fig. 6a). This inconsistency is most likely explained by the amount of template used for the PCR. RNA yields from the LCM-harvested

Fig. 2 Alignment of the nucleotide sequence of the 3′ UTRs of *BEL5* (**a**), *BEL29* (**b**), and *BEL30* (**c**) from *S. tuberosum* (*St*), *S. palustre* (*Sp*), and *S. etuberosum* (*Se*). The lengths of these respective UTRs are listed in Table 2

a

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StBEL5     2212 TGAATACCAGAAAGTCTCGTATTGATAGCTGAAAAGATA  2250
SpBEL5     2225 TGAATACCAGAAAGTCTCGTATTGATAGCTGAAAAGATA  2264
SeBEL5     2225 TGAATACCAGAAAGTCTCGTATTGATAGCTGAAAAGATA  2264

AAAGGAAGTTAGGGATACTCTTATATTGTGTGAGGCCTTCTGGCCCAAGTCGGAGGACCC 2310
AAAGGAAGTTAGGGATACTCTTATATTGTGTGAGGCCTTCTGGCCCAAGTCGGAGGACCC 2324
AAAGGAAGTTAGGGATACTCTTATATTGTGTGAGGCCTTCTGGCCCAAGTCGGAGGACCC 2324

AATTTGATACAACCTATCATAGGAGAAAAGAAGTGGAGACTAAATTAAAGTAACAAAATT 2370
AATTTGATACAACCTATCATAGGAGAAAACAAGTGGAGAC -AAA- -A- - GTAACAAAATT  2379
AATTTGATACAACCTATCATAGGAGAAAACAAGTGGAGAC -AAA- -A- - GTAACAAAATT  2379

TTAAAGCACACTTTCTAGTATATATACTTCTTTTTTTTATAGTATAGAAAAGAAGAGATT  2430
TTAAAGCACACTTTCTAGTATATAAACTTCTTTTTTTTATAGTATAGAAAAGAAGAGATT  2439
TTAAAGCACACTTTCTAGTATATAAACTTCTTTTTTTTATAGTATAGAAAAGAAGAGATT  2439

TTGTGCTTTAGTGTATAGATAGAGTC - TACTTAGTATAGGTTATACTTCTAGTTCCTTGA  2490
TTGTGCTTTAGTGTATAGATAGAGTCCTACTTAGTATAGGTTATACTTCCAGTTCCCTGA  2499
TTGTGCTTTAGTGTATAGATAGAGTCCTACTTAGTATAGGTTATACTTCCAGTTCCCTGA  2499

GAAGATTGATACA –ACTAGTAGTATTTTT- TTT-CTTTT- GGGTT- - - - - - G- - - GCTTG  2550
GAAGATTGATACGTACAACTAGTATTTTTCTTTTCTTTTTGGGTTTAAATAGCTTGCTTG  2559
GAAGATTGATACGTACAACTAGTATTTTTCTTTTCTTTTTGGGTTTAAATAGCTTGCTTG  2559

GAGTACTATTTTAAGTTA –T-TGGAAACTAGCTATAGTAAATGTTGTAAAGTTGTGATAT  2610
GAGTACTATTTTAAGTTAATATGGAAACTAGCTATAGTA TATGTTGTGAAGTTGTGATAT  2619
GAGTACTATTTTAAGTTAATATGGAAACTAGCTATAGTA TATGTTGTGAAGTTGTGATAT  2619

TGTTCCTCTCAATTTGCATATAATTTGAAATATTTTGTACCTACTAGCTAGTCTCTAAAT  2670
TGTTCCTCTCAATTTGCATATAATTTGAAATATTTTGTACCTACTAGCTAGTCTCTAAAT  2679
TGTTCCTCTCAATTTGCATATAATTTGAAATATTTTGTACCTACTAGCTAGTCTCTAAAT  2679

TATGTTTCCATTGCTTGTAATTGCAATTTTATTTGAATTTTGTGCTATCATTATTAGATTAGC 2733
TATGTTTCCATTCCTTGTAATTGCAATTTTATTTGAATTTTGTGCTATCATTATTAGATTAGC 2742
TATG                                                          2683
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b

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StBEL29  TCACAAAAACAAAAACAGGTTTTGGCAACAGACAAACTTCTGTCGCTAAA  1754
SpBEL29  TCACAAAAACAAAAACAGGATTTAGCGACAGACAAACTTCAGTTGCTAAA  1756
SeBEL29  TCACAAAAACAAAAACAGGATTTAGCGACAGACAAACTTCAGTTGCTAAA  1756

CAAGGACATGATTTAGCGACAGATAACTTCAGTCGCTAA -C-T - - - - - T- -A- - - GCGA  1801
CAAGAACATGATTTAGTGACAGATAACTTCCGTCGCTAAACATGAAAATGTATTAGTGA  1815
CAAGAACATGATTTAGTGACAGATAACTTCCGTCGCTAAACATGAAAATGTATTAGTGA  1815

CTGAAAACTTCTGTCGCTAAGCATGAACATGTATTAGCGACATACAGTATGCAACTGTA  1860
CTGAAAACTTTTGTCGCTAAACATGAACATGTATTAGCGACATAC - G-A- - C- - - TGTA  1867
CTGAAAACTTTTGTCGCTAAACATGAACATGTATTAGCGACATAC - G-A - -C- - - TGTA  1867

TGTCACTAAACAAGAACATGATGAATTAGTGACGGACAACTTCTGTCGCTAAACAACAA  1919
TGTCGCTAAACAAGAACATGATGAATTAGCGACTGACAACTTCTGTCGCTAAACAACAA  1926
TGTCGCTAAACAAGAACATGATGAATTAGCGACTGACAACTTCTGTCGCTAAACAACAA  1926

AAAAAAATCCATGTTTT-AGTATATTGTTTCTCATTCTATCATATCATGGTAGTGTAAA  1977
AAAAA - -TC -ATCTTTTTAGTATATTGTTTCTCATTCTATCAT- - CATGGTAGTGTAAA  1980
AAAAA - -TC -ATCTTTTTAGTATATTGTTTCTCATTCTATCAT- - CATGGTAGTGTAAA  1980

GAATCAAGAAACAAGTTTTACATAGTAACAGTCTTTATACATTGGAG  2024
GAATCAAGAAACAAGTTTTACATAGTAACAGTCTTTATACATTGGAG  2027  SpBEL29
GAATCAAGAAACAAGTTTTACATAGTAACAGTCTTTATACATTGGAG  2027  SeBEL29

ATGAAGAACCATTTAAGTTCTTCAAAATAGATAGATAGATTTTCTAGGTTACTTCTANA 2083

AGATATATATATGGTTGAGGGTTTGTATATT  2114  StBEL29
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c

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StBEL30  TTTTG- -TATGTGTTGTAGAATTAAACTGCAAGTTTTGAGTACATCAACATTCATCTTC  2033
SpBEL30  TTTTGTATATGTGTTGTAGAATTAAACTGCAAGTTTTGAGTACATCAACATTCATCTTC  1998
SeBEL30  TTTTGTATATGTGTTGTAGAATTAAACTGCAAGTTTTGAGTACATCAACATTCATCTTC  1998
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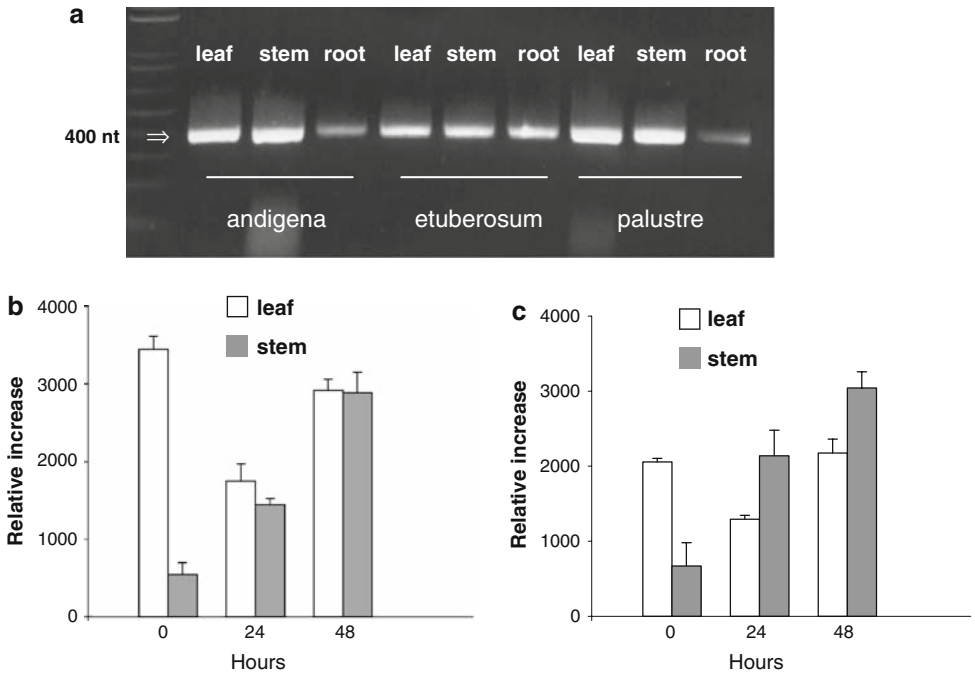

Fig. 3 RT-PCR products for *BEL5* from RNA extracted from leaf, stem, and roots of *Solanum tuberosum* ssp. *andigena*, and two nontuber-bearing species, *S. etuberosum* and *S. palustre* (**a**). Gene-specific primers were used for each species and are listed in Table 1. The expected PCR product size for the *BEL5* RNA is 375 nt. The effect of wounding on RNA accumulation in the leaves and stems of two nontuber-bearing potato species, *S. etuberosum* (**b**) and *S. palustre* (**c**). On intact plants, leaves were wounded with a hemostat and stems with a razor blade and samples were harvested 24 and 48 h after wounding. RNA was extracted and one-step RT-PCR was performed using 20 ng of total RNA, and gene-specific primers for *BEL5* (Table 1). The internal control for PCR reactions was rRNA. All PCR reactions were standardized and optimized to yield product in the linear range. Homogenous PCR products were quantified using ImageJ software (Abramoff et al. 2004) and normalized using the rRNA values. Standard errors of the means of the three biological replicates are shown

cells totaled approximately 30 ng and 2–3 ng of RNA template were used per RT-PCR (Yu et al. 2007). In the current study, RNA yields ranged from 280 to 840 ng/200 µl of harvested sap (Fig. 5) and approximately 70 ng of RNA extracted from sap was used as template for the *StBEL14* assay (Fig. 6a).

To determine if *BEL5*-like RNAs are present in phloem RNA from nontuber-bearing solanaceous species, RT-PCR was again performed on RNA extracted from sap collected after 30 min of bleeding. *BEL5*-like RNA was detected in sap RNA from *S. etuberosum* and *S. lycopersicum* cv. BHN (Fig. 6b). *BEL5* RNA was also detected in the phloem sap of *S. palustre* (data not shown). Phloem sap from tobacco (Nt) stems could not be obtained from stem excisions so primary midveins harvested from the abaxial (lower) side of the leaf blade were used instead. These prominent veins protrude 2–3 mm from the lower side of the leaf blade and were relatively easy to excise with a razor blade without any leaf mesophyll contamination. A large proportion of *BEL5* RNA was detected in RNA from these veins relative to the amount detected in RNA from leaf (Fig. 6b, Nt). The lower section of midveins contains, in order, starting from the lamina, xylem, phloem, and collenchyma tissue (Esau 1977). Consistent with these results, in potato, the foliar midveins are the primary source of *StBEL5* promoter activity (Chatterjee et al. 2007).

Discussion

KNOTTED1-like RNAs in the phloem

The presence of several *KNOTTED1*-like RNAs in phloem-enriched sap of potato, including both class-I and -II types, was confirmed in this study. Previous work using laser capture microdissection and in situ hybridization showed that the RNA of *POTH1*, a KNOX protein that interacts with StBEL5, could be detected in phloem cells of the stem (Yu et al. 2007) and the stolon tip (Rosin et al. 2003). The presence of a mRNA in the phloem sap, however, does not prove that it is transported. *CmSTMP*, a *KNOTTED1*-like gene involved in meristem maintenance, was identified in the phloem sap of *Cucurbita maxima* (pumpkin) but its movement into a cucumber scion could not be confirmed (Ruiz-Medrano et al. 1999). Other *KNOTTED1*-like transcripts have been transported across a graft union. A fusion of a *phosphofructokinase-knotted1* transcript of tomato, *PFP-LeT6*, moved across a graft and induced a develop-

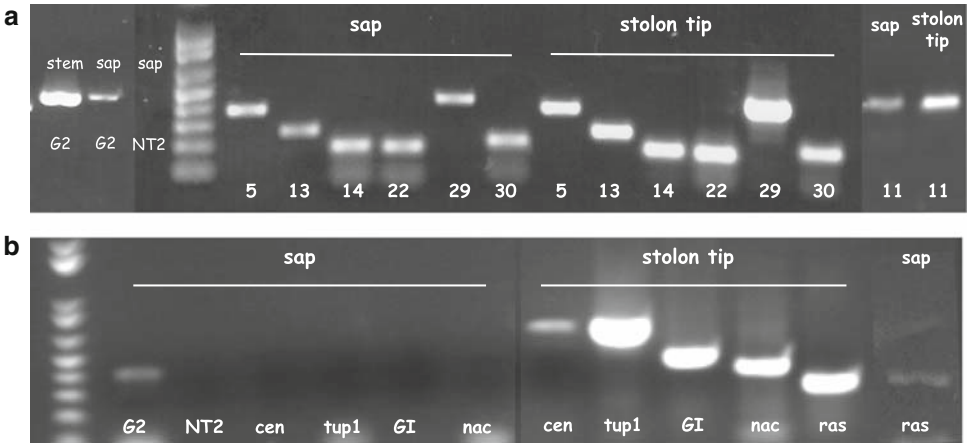

Fig. 4 RT-PCR products from RNA extracted from either stem sap or stolon tips for several *BEL1*-like RNAs (**a**, designated 5, 13, 14, 22, 29, 30 and 11) or from several RNAs detected in stolon tips (Hannapel 2007) during an early stage of tuber formation (**b**). Sap from the lower stem of potato plants was harvested in 200 µl aliquots. Total RNA was extracted and used as the RT-PCR template for detecting tissue-specific transcripts. *G2* is RNA for a phloem-specific transcription factor (Zhao et al. 2005) and *NT2* is a positive control for root xylem cells (Nazoa et al. 2003). Source plants for these experiments were grown under short-day conditions (8 h light, 16 h dark). Gene-specific primers listed in Table 1 were used in the PCR reactions. *Cen centroradialis*-like RNA, *tup1* a DNA-binding protein, *GI Gigantea*, *nac* ubiquitous family of transcription factors (NAM/ATAF1, 2/CUC2), *ras* ras-related protein, an extensive family of small GTPases that regulate cell growth and differentiation

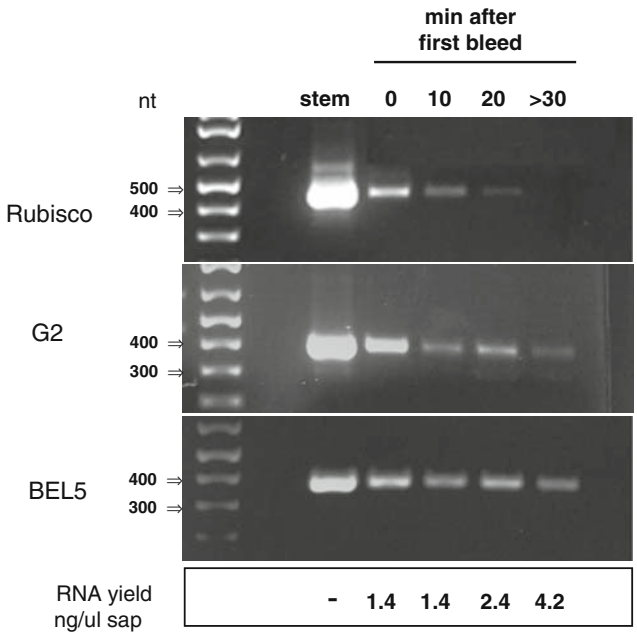

Fig. 5 RT-PCR products from RNA extracted from sap harvested from excised stems over time intervals from the onset of exudate flow. For the sap harvests, 200 µl of sap was collected from the same set of several plants during each time interval: immediately upon bleeding (*0*), 10–20 min from the onset of bleeding (*10*), 20–30 min (*20*), and after 30 min from the onset of stem bleeding (*>30*). Gene-specific primers (Table 1) for the small subunit of rubisco, the phloem-specific RNA, *G2* (Zhao et al. 2005), and *StBEL5* were used in the PCR reactions. The expected sizes of the PCR products were 450, 390, and 375 nts, respectively. Stem RNA extracted from the same plants was used as a control

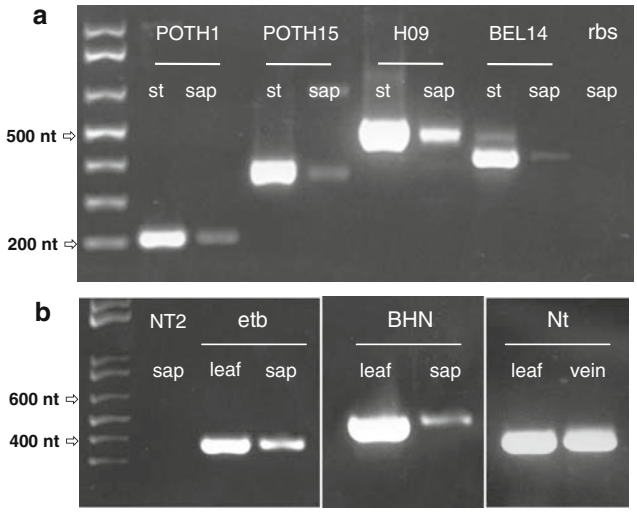

Fig. 6 RT-PCR products from the RNA extracted from stem sap harvested after 30 min from the initial bleed for three *KNOTTED1*-like mRNAs and *StBEL14* in *S. tuberosum* ssp. *andigena* (**a**) and for *BEL5* from three nontubering-bearing species (**b**). Gene-specific primers were used with each PCR reaction (Table 1). *POTH1* and *−15* are class-I *KNOX* genes (Rosin et al. 2003) and *H09* is a class-II type (Reiser et al. 2000). The expected sizes of the RT-PCR products in **a** for *POTH1*, *POTH15, H09,* and *BEL14* are 210, 361, 492, and 424 nts, respectively. For the *BEL5* products in **b**, the expected sizes are 375, 500, and 400 nts for etb, BHN, and Nt, respectively. Stem (**a**) or leaf (**b**) RNA extracted from the same plants were used as positive controls. Phloem sap from tobacco (**b**, Nt) stems could not be obtained so primary midveins harvested from the abaxial side of the leaf blade were used as source tissue instead. *NT2* is a positive control for root xylem cells and is not present in phloem cells (Nazoa et al. 2003). *St* Stem, *rbs* rubisco small subunit, *etb S. etuberosum*, *BHN S. lycopersicum* cv. BHN, *Nt Nicotiana tabacum* var. Petit Havana. Sequencing of randomly selected bands was used to confirm the identity of PCR products

mental phenotype (Kim et al. 2001). The best examples of *KNOX* RNA movement, however, are in association with its protein through the plasmodesmata of tobacco mesophyll cells (Lucas et al. 1995) and from cell-to-cell using a trafficking assay coupled to trichome rescue in *Arabidopsis* (Kim et al. 2005). In the latter study, the KNOX homeodomain of the protein promoted intercellular trafficking of both the KNOX protein and its associated mRNA. In this report, the observation that *KNOTTED1*-like mRNAs of potato are present in the phloem at the same time and location as the mRNAs from their protein partners of the BEL1-like family is intriguing. As previously reported (Chen et al. 2004), the tandem complex consisting of both protein types was necessary for regulating transcriptional activity in a target gene that affected tuber formation.

What is the function of *BEL5* RNAs in the phloem of nontuber-bearing species?

Clearly, *BEL1*-like genes function in a wide variety of roles in plant development and metabolism. *StBEL5* is unique in that it functions as a long-distance signal to regulate vegetative growth in a specialized underground organ, the tuber (Banerjee et al. 2006). But what is the function of *BEL5*-like RNAs in the phloem of nontuber-bearing species? Two *BEL5* RNAs, from tobacco and tomato, even contain conserved sequence motifs in their respective 3′ UTRs that are also present in *StBEL5* (data not shown). With *StBEL5* RNA, the 3′ UTR has been implicated in mediating mobility (Banerjee et al. 2006). Based on the wound-induction pattern previously described (Chatterjee et al. 2007) and verified in the current study, it is plausible that *BEL5*-like genes function as a phloem defense signal responsive to mechanical or insect damage. A *BEL1*-like gene from rice, *OsBIHD*1, was identified that functions in disease resistance and pathogen defense (Luo et al. 2005). A protein partner of BEL1, Brevipedicellus, a KNOTTED1-like transcription factor of *Arabidopsis*, regulates several genes involved in lignin biosynthesis (Mele et al. 2003), implying that the BEL/KNOX complex may be involved in rebuilding cell walls in the vascular tissue after wounding or damage from insect predation.

Harvesting phloem RNAs from potato

Plant species from the Cucurbitaceae have been studied extensively for phloem analysis because of their propensity for releasing phloem-abundant sap from excised stems (Ruiz-Medrano et al. 1999). For *Ricinus communis,* phloem sap was collected from the cotyledon for 15 min following the excision of the seedlings (Doering-Saad et al. 2006). With *Brassica napus*, phloem samples were obtained by making small punctures with a hypodermic needle into inflorescence stems of 8- to 10-week-old plants. The first flowing droplet was discarded and the subsequent exudate was collected into sample buffer (Giavalisco et al. 2006). All of these collection techniques yielded phloem-enriched RNA populations.

After preliminary work, phloem-enriched sap was effectively harvested from excised potato stems in this study. By allowing flow to occur for up to 30 min, contaminants from wounded cells were reduced to a minimum. Even though it is assumed that this harvested sap contained xylem flow, based on previous analyses demonstrating that xylem sap contains no RNA (Buhtz et al. 2008) and the results of this study (Figs. 4, 5), it may be concluded that the harvested sap described here contains a phloem-enriched RNA population. In summary, these results indicate the presence of numerous transcripts from the TALE superfamily of transcription factors in phloem cells. The process of transporting full-length mRNAs through the phloem as long-distance signals seems to be much more extensive than previously assumed. What we know so far about this dynamic information system appears to be just the tip of the iceberg.

The following GenBank accession numbers have been assigned: EU686384 for *SeBEL5*, EU686378 for *SeBEL14*, EU686385 for *SeBEL29*, EU686379 for *SeBEL30*, EU686380 for *SpBEL5*, EU686381 for *SpBEL14*, EU686382 for *SpBEL29*, and EU686383 for *SpBEL30*.

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