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Accumulation of BEL1-like transcripts in solanaceous species

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Accumulation of *BEL1*-like transcripts in solanaceous species

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CHAPTER 1. GENERAL INTRODUCTION

Plant breeders make crucial decisions in plant breeding programs, which involve the selection of parents for cultivar development. Factors such as the trait to be improved, understanding how the trait is transmitted, and identifying potential germplasm must be taken into consideration. The potato (*Solanum tuberosum*) is the world's fourth most important food crop in terms of global annual production (Fernie and Willmitzer 2001). Tuber formation is summarized by retardation of stolon elongation, thickening of the sub-apical region of the stolon tip via cell enlargement and cell division, and increased storage of proteins and carbohydrates at the site of tuber development (Verhees et al. 2002). It should be noted that several commercial varieties of potato are polyploid, and genetic improvement has been slower than other agronomically important crops, such as corn, rice, or soybean. As a consequence, a collection of genetic mutants for potato tuberization is not available. Ironically, improving desired traits of potato cultivars can be quickly achieved using backcross programs that involve plant introductions and/or related species (Tek et al., 2004).

Recently, the non tuber-bearing (NTB) species *S. palustre* (ACN no.: PI 558259) has been utilized for its tuber soft rot resistance and early blight resistance genes (Tek et al., 2004). *Solanum etuberosum* (ACN no.: PI 498311) is also a NTB species that has been implemented in breeding programs for having resistances to three major viruses of potato: potato leafroll virus (PLRV), potato viruses X (PVX), potato virus Y (PVY) and exhibiting resistance to green peach aphid, a primary insect vector of PLRV and PVY (Novy et al. 2007). Both of these species reproduce from seed, while tuber-bearing (TB) species can be propagated using seed or tubers. It might appear that tuber-bearing and non tuber-bearing plants aren't closely related but hybrids can be created using somatic hybridization.

Transcription factors (TF) are proteins that act as developmental switches by binding to the DNA of specific genes to regulate their expression. An important family of TFs involved in regulating the developmental events in apical meristems is the knox (knotted-like homeobox) gene family. Knox genes belong to a group of TFs known as the three-amino acid loop extension (TALE) superclass and are sub-divided into two classes (Burglin et al. 1997). Class I *knox* genes are expressed in shoot apical meristems, while class II genes have a more diverse expression pattern (Reiser et al. 2000).These TFs are unique due to a very high level of sequence conservation in the DNA-binding region, designated the homeodomain (HD), and consists of three α-helices (Kerstetter et al.1994). There are numerous TFs from plants and animals in the TALE superclass, but the two main groups in plants are the KNOX and BEL types and protein members of these two groups have been identified that physically interact.

Previous research in our lab suggests a *BEL1*-like TF, *S. tubersosum* BEL5, plays a role in tuber formation by forming a heterodimer with a *KNOTTED1*-like TF, POTH1, and binding to the promoter of ga20 *oxidase1* to down-regulate its giberellic acid activity (Chen et al. 2004). Subsequently cytokinin levels are enhanced and the result of this pathway is enhanced tuber formation (Banerjee et al. 2006). It is not known if BEL5 is functional in NTB species, but studying RNA accumulation patterns in the leaf, stem, and roots will help answer this question.

The objectives of this study are to: a) Determine if *BEL5* and *POTH1* are present in NTB species; and b) Analyze the expression of *BEL1*-like and *KNOTTED1*-like transcripts in NTB species for sequence and functional differences as compared to tuberizing species.

Thesis Organization

The thesis consists of one journal article preceded by a General Introduction and followed by a General Conclusion and a Literature Cited section. The article is formatted according to the requirements of the journal. “Accumulation of *BEL1*-like transcripts in solanaceous species” has been accepted for publication in *Planta*. Brian Anthony Campbell was the primary investigator for this work under the supervision of Dr. David J. Hannapel and is the first author.

CHAPTER 2. ACCUMULATION OF *BELL1*-LIKE TRANSCRIPTS IN *SOLANACEOUS* SPECIES

A paper accepted for publication in *Planta*

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SUMMARY

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 contamination from RNA arising from wounded cells, *KNOTTED1*-like RNAs were detected in phloem-enriched sap of potato and *BEL5* RNA

was detected in sap collected from two closely related nontuber-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.

INTRODUCTION

As 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). Cosuppression 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 (Burglin 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.

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 entire 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 demonstrated that the promoter of St*BEL5* was activated in response to wounding in stems but 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 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 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 wounded cells of the cut surface of the stem, RNA was extracted from 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 by 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 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 800 ng/200 ul of harvested sap (Fig. 5) and approximately 70 ng of RNA extracted from sap was used as template for the *StBEL14* assay of 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 developmental 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 minutes, 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 and 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.

MATERIALS & 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 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 two 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 a growth chamber at approximately 21°C. Sap was collected from stock plants after they were grown for approximately three 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 one 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 the harvest in Fig. 4, at least 200 µl of sap was collected across the stem with a 0.2 ml Pipetman and stored on ice. For the analysis of Fig. 5, 200 ul of sap was collected during each time interval: immediately upon bleeding, 10 to 20 min from the onset of bleeding, 20 to 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 sec, 0.2 ml chloroform was added, and the sample was again vortexed for 30 sec. The sample was then centrifuged (12K rcf) for 15 min @ 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 template used varied among reactions (20 to 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 sec, 54 to 56 °C for 30 sec, 68°C for 30 sec. *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 *S. 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 by using the Basic Local Alignment Search Tool (BLAST). The percent nt matches were determined by a comparison to the known *StBEL* sequences (Table 2).

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*.

Wounding experiment

S. etuberosum and *S. palustre* stock plants were propagated from 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 soil level superficially with a clean razor blade or by 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 sec, 56°C for 30 sec, 68°C for 30 sec. 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 sec, 56°C for 30 sec, 68°C for 30 sec. Homogenous PCR products were quantified by using ImageJ software (Abramoff et al. 2004) and normalized by 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.

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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	2742 (Sp)
BEL5-1C-R	CTAGTTGTATCAATCTTCTCAAGG	
BEL14-3A-F	GTGATCATGGTCCTTCGTCTTC	1606
BEL14-3B-R	TCATGAAGAAGTTGTGCCC	
BEL29-4A2-F	CAAGGGCTTTCACTTAGC	2028
BEL29-4F-R	CTCCAATGTATAAAGACTGTTACTATG	
BEL30-2A-F	GATGGCGACTTATTTTCCTAGTCC	1998
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	

Table 2. A comparison of cDNA clones from the *BEL1*-like family of transcription factors in two nontuber-bearing *Solanum* species. Se, *Solanum etuberosum*; Sp, *Solanum palustre;* St, *Solanum tuberosum*. The percent nt match is to the *BEL1*-like ortholog in *Solanum tuberosum* (Chen et al. 2003). NA, data not available.

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

Figure Legends

Figure 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*.

Figure 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.

Figure 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 hr 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 by using ImageJ software (Abramoff et al. 2004) and normalized by using the rRNA values. Standard errors of the means of the three biological replicates are shown.

Figure 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 ul 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 use 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.

Figure 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 ul of sap was collected from the same set of several plants during each time interval: immediately upon bleeding (0), 10 to 20 min from the onset of bleeding (10), 20 to 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.

Figure 6. RT-PCR products from 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 panel **a** for *POTH1*, *POTH15, H09,* and *BEL14* are 210, 361, 492, and 424 nts, respectively. For the *BEL5* products in panel **b** for etb, BHN, and Nt, the expected sizes are 375, 500, and 400 nts, 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.

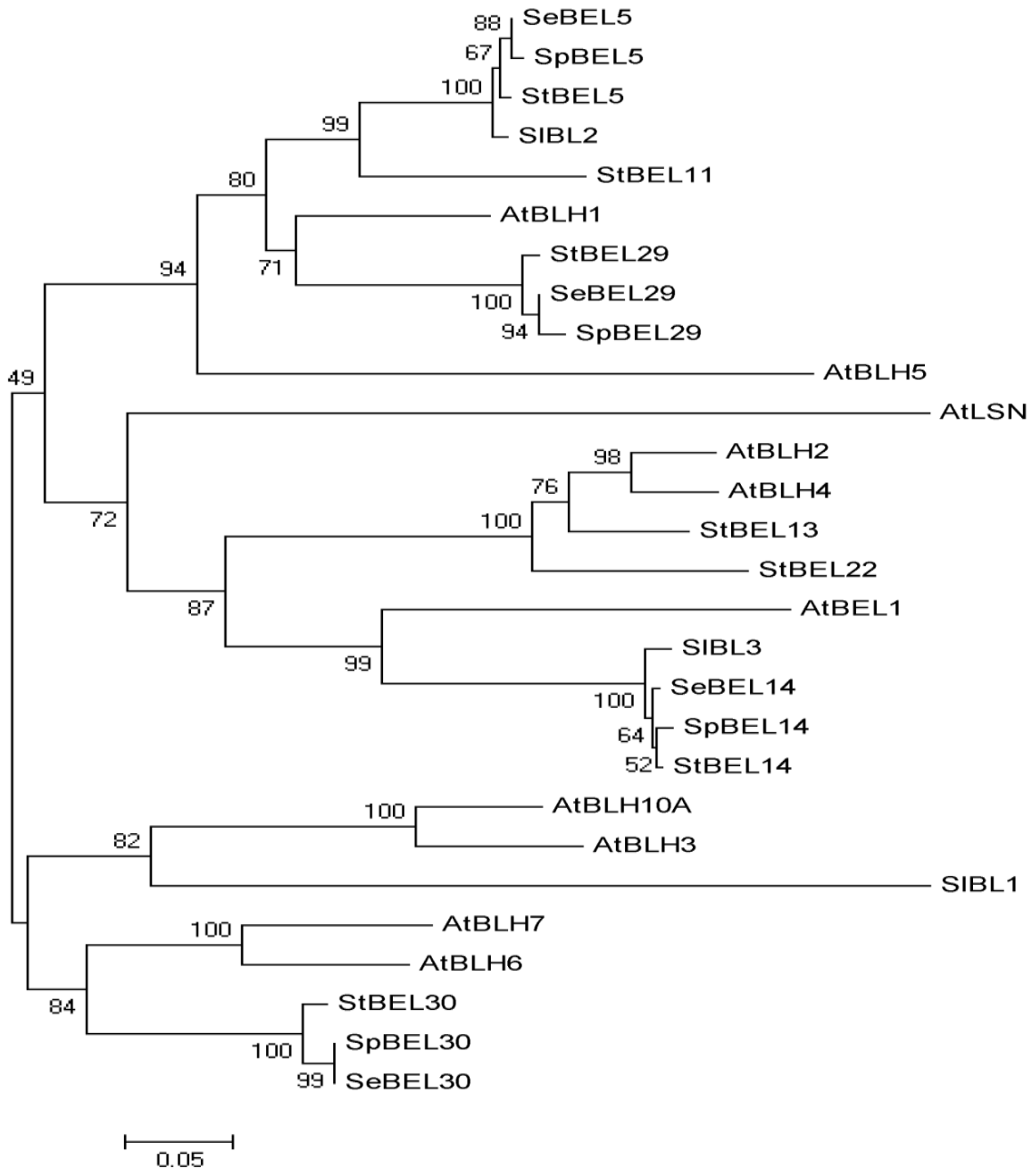

SeBEL5
SpBEL5
StBEL5
SlBL2
StBEL11
AtBLH1
StBEL29
SeBEL29
SpBEL29
AtBLH5
AtLSN
AtBLH2
AtBLH4
StBEL13
StBEL22
AtBEL1
SlBL3
SeBEL14
SpBEL14
StBEL14
AtBLH10A
AtBLH3
SlBL1
AtBLH7
AtBLH6
StBEL30
SpBEL30
SeBEL30
88
67
100
99
80
94
71
100
94
49
98
76
72
100
87
99
100
64
52
100
82
100
84
100
99
0.05

Figure 1.

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

Figure 2a.

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*

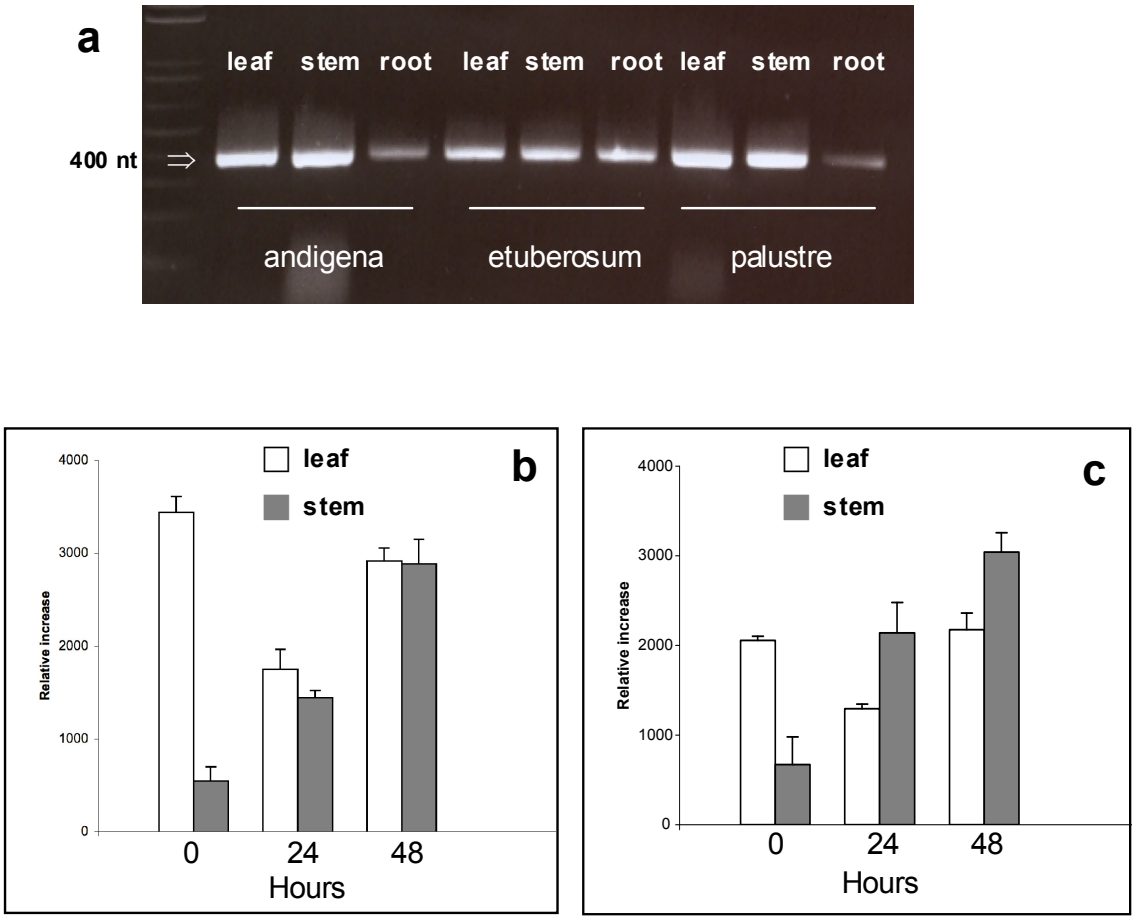
ATGAAGAACCATTTAAGTTCTTCAAAATAGATAGATAGATTTTCTAGGTTACTTCTANA 2083

AGATATATATATGGTTGAGGGTTTGTATATT 2114 *StBEL29*

Figure 2b.

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

Figure 2c.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leaf stem root leaf stem root leaf stem root
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andigena
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palustre
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stem
Relative increase
4000
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stem
Relative increase
4000
3000
2000
1000
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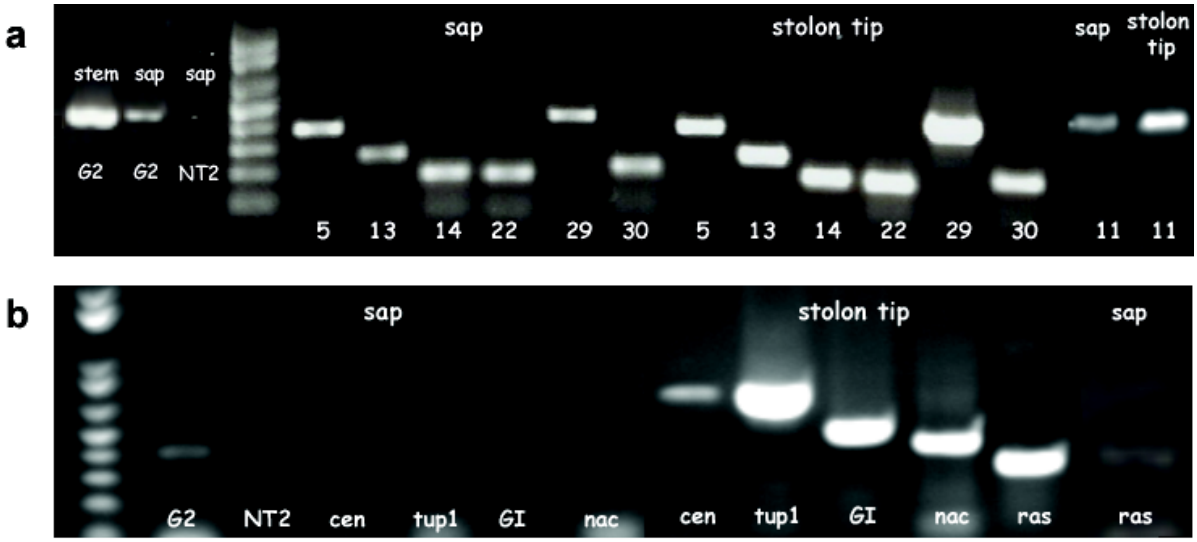
24
48
Hours

Figure 3.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sap
stolon tip
sap
stolon tip
stem
sap
sap
G2
G2
NT2
5
13
14
22
29
30
5
13
14
22
29
30
11
11
b
sap
stolon tip
sap
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NT2
cen
tup1
GI
nac
cen
tup1
GI
nac
ras
ras

Figure 4.

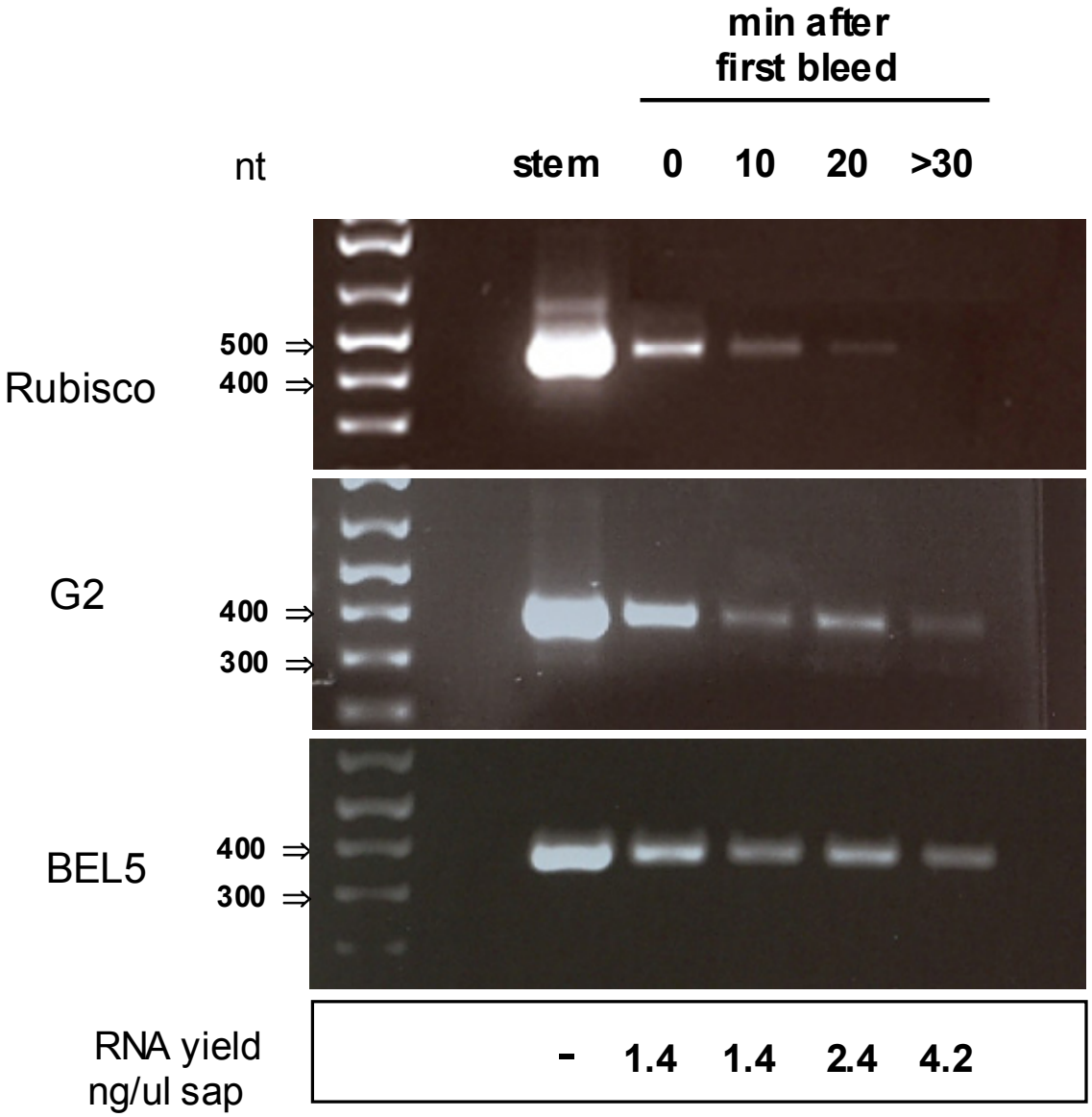
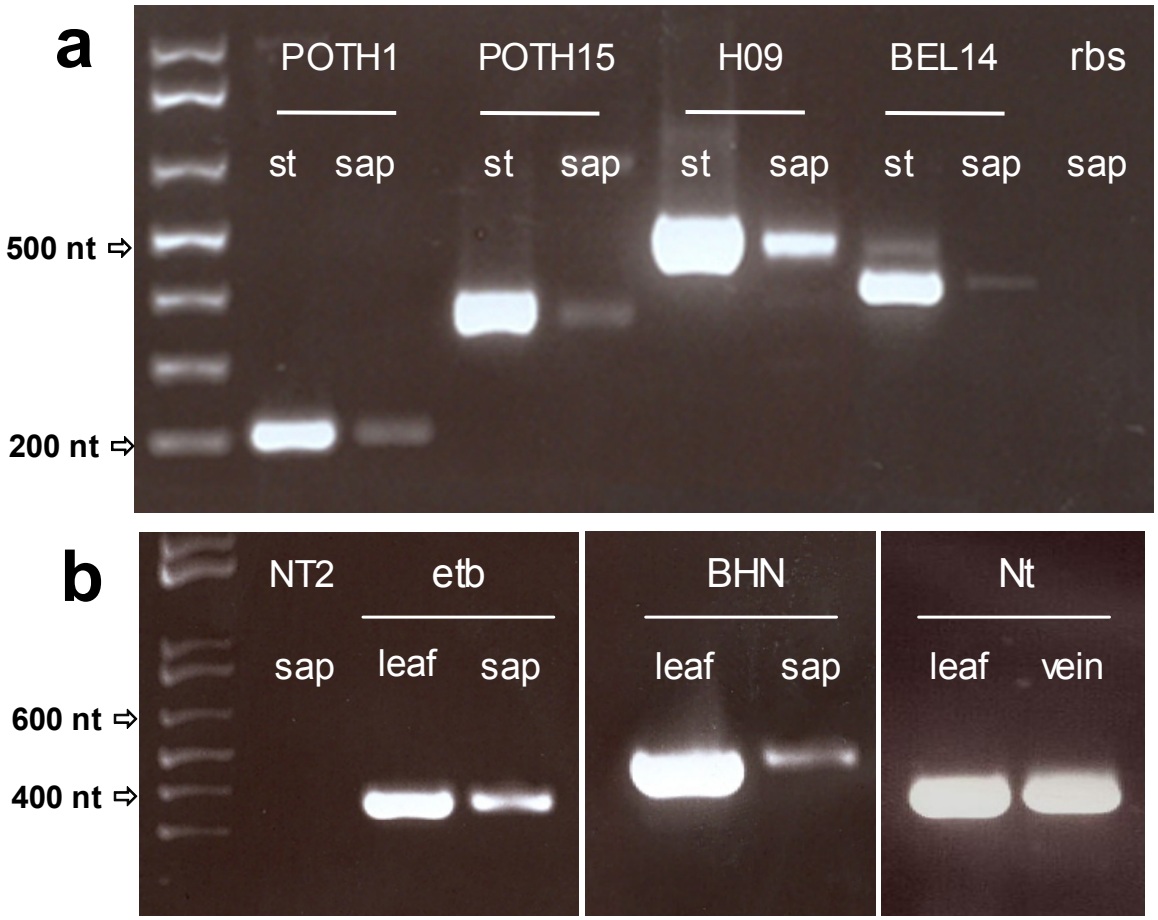

min after
first bleed
nt
stem
0
10
20
>30
Rubisco
500
400
G2
400
300
BEL5
400
300
RNA yield
ng/ul sap
-
1.4
1.4
2.4
4.2

Figure 5.


a
POTH1
POTH15
H09
BEL14
rbs
st
sap
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st
sap
sap
500 nt
200 nt
b
NT2
etb
BHN
Nt
sap
leaf
sap
leaf
sap
leaf
vein
600 nt
400 nt

Figure 6.

CHAPTER 3. GENERAL CONCLUSIONS

In this study, eight homologs of the BEL family were identified in two NTB species of potato. Analyses utilizing BLAST and phlylogenetic software revealed high sequence similarity at the transcript and protein level to their tuber-bearing counterparts. Therefore, these novel potato BELs were named SeBELs and SpBELs, indicating BELs from *S. etuberosum* and *S. palustre*, respectively. Consistent with previous work from Chen et al. 2003, the new BELs clustered with their tuber-bearing counterparts when a dendrogram was constructed using identical parameters. In addition, *SeBEL5* and *SpBEL5* expression was consistent with the induction pattern of *StBEL5* in wounded stems but not in leaves over a forty-eight hour time period (Chatterjee et al. 2007). The results of this study indicate the presence of numerous transcripts from the TALE superfamily of transcription factors in phloem cells of several solanacceous species.

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